KISS1 GENE EXPRESSION AND THE EFFECTS OF KISSPEPTIN DURING PUBERTAL DEVELOPMENT IN THE EWE LAMB

A Thesis

by

JEREMY SCOTT REDMOND

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Committee,	Marcel Amstalden
Committee Members,	Gary L. Williams
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ABSTRACT

Kiss1 Gene Expression and the Effects of Kisspeptin During Pubertal Development in the Ewe Lamb. (December 2010)

Jeremy Scott Redmond, B.S., The Pennsylvania State University

Chair of Advisory Committee: Dr. Marcel Amstalden

Increased pulsatile release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) is critical for pubertal initiation of ovarian cycles in female mammals. Kisspeptin, a peptide product of the *Kiss1* gene, is required for normal puberty. In Experiment 1, ovariectomized ewe lambs bearing subcutaneous estradiol implants were used to investigate *Kiss1* gene expression in the preoptic area (POA) and hypothalamus during pubertal maturation of the reproductive neuroendocrine system. Brain tissue was collected from ewes at 25, 30, and 35 wk of age (n=6/group). Patterns of LH release in circulation were determined on the day before euthanasia and cells containing *Kiss1*-mRNA were identified by in situ hybridization. Mean concentrations of LH and the frequency of LH pulses increased ($P < 0.01$) as ewe lambs matured. In the POA/Periventricular area (PEV), the number of *Kiss1*-expressing cells was greater ($P < 0.04$) in 30- and 35-wk-old than in 25-wk-old ewe lambs. In the arcuate nucleus (ARC), although no significant changes in number *Kiss1*-expressing cells were observed among age groups, the number of *Kiss1* cells increased ($P < 0.02$) with increased frequency of LH release. This resulted in greater ($P < 0.01$) number of *Kiss1* cells in the ARC of ewes

demonstrating elevated frequency (> 6 pulses/12 h) of LH pulses. In Experiment 2, 28-wk-old ewe lambs were used to determine the effects of intermittent injections of kisspeptin on the release of LH and stimulation of gonadal function in peripubertal ewe lambs. Ewe lambs were treated intravenously with saline (Controls; n=6) or kisspeptin (n=6) hourly for 24 h. Blood samples were collected throughout the experiment for hormone analysis. Kisspeptin-treated lambs had greater ($P < 0.02$) mean circulating concentrations of LH, and frequency and amplitude of LH pulses than controls. Four of six kisspeptin-treated ewe lambs exhibited LH surge and luteal activity in response to treatments. However, onset of regular estrous cycles was not established immediately following kisspeptin-induced ovulation and no difference in age at onset of puberty was observed between groups. In conclusion, activation of the hypothalamic kisspeptin system may support elevated episodic release of LH critical for establishment of normal estrous cycle during pubertal development.

DEDICATION

To my parents, Michael and Lori,

whose undying love and support help me accomplish anything I set my mind to.

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CHAPTER I

INTRODUCTION

A well characterized endocrine event that precedes the onset of reproductive cycles in females of mammalian species is the increase in the frequency of luteinizing hormone (LH) release during the peripubertal period [1-3]. Sustained elevation of circulating LH supports the final stages of follicular growth, steroidogenesis and ultimately, ovulation. The development of a pulsatile pattern of release of LH during puberty is believed to result from the activation of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA) and hypothalamus [4] and is required for normal reproduction in mammals [5]. However, the neuroendocrine mechanisms leading to the elevated frequency of GnRH/LH release prior to the onset of regular reproductive cycles are not well understood. Changes in the sensitivity to inhibitory feedback actions of estradiol seem to play an important role [1]. In addition, the onset of puberty is largely influenced by the availability of food, as undernutrition delays the pubertal increase in the frequency of GnRH/LH release [6, 7]. Therefore, nutritional and metabolic factors interact with gonadal steroids to regulate reproductive maturation.

Recent studies have suggested a critical role for the neuropeptide, kisspeptin, and its receptor, Kiss1r (also known as G-coupled Protein Receptor-54, GPR-54), in the regulation of reproductive function. Kisspeptin is a potent stimulator of GnRH and LH

This thesis follows the style of Biology of Reproduction.

release [8]. The expression of the *Kiss1* gene is regulated by estradiol [9, 10] and responsive to nutritional restriction [11], suggesting that kisspeptin neurons may be involved in integrating estradiol negative feedback and metabolic influences on GnRH neuronal activity. Mutations in *Kiss1* or *Kiss1r* genes result in hypogonadotropic hypogonadism and failure to attain puberty [12, 13]. Moreover, *Kiss1* and *Kiss1r* gene expression increases during reproductive maturation in monkeys [14], rats [15], and mice [16]. However, it is unclear whether changes in *Kiss1* expression occur in distinct neuronal populations during the pubertal transition to elevated episodic release of LH. Therefore, the objectives of these studies were: 1) to examine *Kiss1* gene expression in the POA and hypothalamus during pubertal maturation of the reproductive neuroendocrine axis in ewe lambs, and 2) to determine the effects of intermittent injections of kisspeptin on the release of LH and stimulation of gonadal function in peripubertal ewe lambs.

CHAPTER II

LITERATURE REVIEW

Neuroendocrine Basis of Puberty

Puberty is a process of physiological changes that supports individuals to acquire the ability to reproduce. In mammalian females, the activation of the hypothalamic-pituitary-gonadal axis, and consequent enhancement of folliculogenesis and steroidogenesis, leads to the onset of puberty [17-19]. The onset of puberty can be characterized by the initiation of frequent pulsatile release of GnRH into the hypothalamic-hypophyseal portal circulation, which in turn stimulates synthesis and release of gonadotropins from the pituitary [17-19]. The peripubertal increase in the frequency of LH release supports final maturation of ovarian follicles and follicular steroidogenesis. Increased concentrations of circulating estradiol induce the preovulatory surge of LH and first ovulation.

There is strong evidence demonstrating that a major limiting factor for the initiation of high frequency, pulsatile release of LH and development of follicles to the preovulatory stage is the lack of appropriate stimulation of gonadotropes by GnRH. Pulsatile administration of GnRH to immature monkeys induces corresponding pulsatile release of LH and supports the development of ovulatory cycles [20], demonstrating that the gonadotropes of prepubertal female monkeys are capable of responding to the GnRH signal. Interestingly, the reproductive endocrine axis reverts to an immature state if GnRH treatment is ceased [20]. In 18-week old ewe lambs, pulsatile administration of

LH (1 injection/h) increased circulating concentrations of estradiol and induced a preovulatory LH surge and ovulation beginning as early as 23 h after initiation of treatments [21]. Moreover, estradiol induces a surge-like release of LH in prepubertal females [22], suggesting that the estradiol positive-feedback system is functional before reproductive maturation is achieved. Thus, the gonadotropes, ovaries and brain of immature females are capable of responding to the appropriate signals to sustain the initiation of ovarian cycles. The lack of appropriate stimulatory signals to sustain GnRH neuronal activity may explain the decreased pulsatile release of GnRH during the prepubertal period. In addition, the presence of increased tone of inhibitory neurotransmitters has also been proposed to contribute to the low frequency of GnRH release prior to the onset of puberty.

It has been suggested that during puberty a decrease in the sensitivity to estradiol negative feedback allows for the increase in circulating LH [23, 24]. In prepubertal ovariectomized rats [25], sheep [1], and heifers [26], circulating concentrations of LH rise after ovariectomy. Estradiol replacement decreases concentrations of LH [1, 25, 26], suggesting that low concentrations of estradiol, characteristic of the prepubertal stage, are inhibitory to the release of LH. However, similar estradiol treatments in ovariectomized females at later stages of reproductive maturation or in mature females fail to inhibit the pulsatile release of LH [1, 25, 26]. Therefore, prepubertal females appear to have greater sensitivity to the inhibitory actions of estradiol compared to mature females. In primates, however, the inhibitory negative feedback mechanism does not seem to develop until later in the juvenile period, because the juvenile hiatus in

gonadotropin secretion observed in the gonad-intact monkey is also observed in gonadectomized monkeys [27].

Nutrition has been demonstrated to have a major effect on timing the onset of puberty in mammals [6, 28]. Undernutrition has been shown to decrease secretion of LH [6, 29, 30]. A similar effect was seen in female rats undergoing excessive exercise [31]. The inhibitory effects of undernutrition on the reproductive axis seem to occur, at least in part, at the hypothalamic level. Exogenous administration of GnRH in chronically undernourished mature ewes results in patterns of LH release similar to those in normal-fed ewes [32]. Undernutrition has been shown to delay puberty until proper nutritional status is reached [32], although in some cases reproductive maturity may occur in animals that adapt to low planes of nutrition [33]. The mechanisms and neuronal pathways involved in the nutritional regulation of GnRH/LH release are not clearly understood, but metabolites, metabolic hormones and neuropeptides seem to be involved [34, 35].

Leptin, a hormone produced mainly by adipocytes, may be a signal to the brain indicating body fat mass and energy reserves [36]. Leptin receptors have not been found on GnRH neurons [37], suggesting that intermediate pathways mediate leptin's effects on GnRH release. The effects of leptin in the hypothalamus may be mediated by neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons [38, 39]. More specifically, it is suggested that NPY mediates the inhibitory effects of undernutrition on GnRH release [40-42]. During puberty, it is proposed that circulating concentrations of leptin are increased in association with increased body fatness [43]. Leptin inhibits

hypothalamic expression of NPY [44]. Consequently, the inhibitory actions of NPY upon GnRH release [41] are minimized and the frequency of GnRH secretion increases.

Growth factors such as transforming growth factor (TGF)-β_1 and TGF–β_2, basic fibroblast growth factor, and epidermal growth factor have also been implicated in the attainment of puberty [45]. These factors, secreted mainly by glial cells, seem to have a role in regulating GnRH release particularly at the level of the median eminence [46]. Recently, Roth et al. [47] used a systems biology approach to examine whether the expression of a network of tumor-related genes is increased in the hypothalamus during puberty. Examples of genes in non-human primates included: 1) *SASH1*, a gene involved in signal transduction; 2) *FLJ22457*, a gene involved in suppression of cell proliferation and promotion of differentiation by regulating the MAPK signaling pathway; 3) *CUTL1*, a transcriptional repressor; and 4) *CCND1*, a gene involved in regulating the cell cycle [47]. Additional examples of genes that increased at the time of puberty in the rat included: 1) *Foxp1*, a gene that promotes the development of the immune system; 2) *Usf2*, a gene that regulates gene transcription (by regulating the FSH receptor gene); and 3) *YY1*, a gene that regulates the expression of imprinted genes necessary for hypothalamic development [47]. Furthermore, *Foxp1* functions in the hypothalamus to promote feed intake and body weight gain and is negatively regulated by leptin [47]. This novel approach demonstrated that genes involved in cell adhesion, cell cycle, immune and stress response, vesicle transport and cell metabolism may interact to regulate maturation of the neuroendocrine system.

Reproduction in some breeds of sheep is regulated by photoperiod as well through mechanisms involving melatonin actions at the level of the hypothalamus [48]. The nocturnal increase in melatonin release from the pineal gland signals photoperiodic changes in the duration of light-dark cycles and is involved in the seasonal regulation of reproduction [49]. Melatonin control of reproductive seasonality in the sheep seems to involve melatonin-sensitive neurons in the premammillary region of the hypothalamus [48, 50] and downstream effects on the pulsatile release of GnRH and LH [49]. Breeding in the seasonal, reproductively mature sheep is generally restricted to the short days of fall and winter [51]. In the ewe lamb, puberty is normally attained during the short-day permissive photoperiod [52]. However, exposure to long days seems to be critical before short days become permissive for the initiation of reproductive cycles in the ewe lamb [53]. Endogenous estradiol interacts with photoperiod to regulate the release of LH in immature ewes [1]. In ewe lambs exposed to short-day photoperiod later during pubertal development, estradiol negative feedback diminishes, and puberty occurs at the expected time. However, if ewe lambs of similar age are exposed to long-day photoperiods, estradiol negative feedback remains heightened and puberty is delayed [1]. Whether the mechanisms involved in estradiol inhibition of LH release in the prepubertal ewe are similar to those regulating seasonal anestrous in the mature ewe [54] remain to be determined. In the mature ovariectomized, estradiol-treated ewe, frequency of pulses of LH gradually increases as day length decreases during the fall, and gradually decreases as the day length increases in the late winter and spring [55].

Link Between Kisspeptin and Reproduction

Kiss1, also known as metastin, was originally discovered in 1996 as a gene with suppressive effects on metastasis of malignant melanoma and carcinoma cells [56]. The *Kiss1* gene encodes a 145 amino acid precursor that is processed by enzymatic cleavage to produce a final peptide product, kisspeptin, that may vary in length depending on the cleavage site. All kisspeptin forms contain a similar carboxyl terminus that is related to the RF-amide peptide superfamily [57]. The shortest kisspeptin form with characterized biological activity is the decapeptide, kisspeptin-10 [57, 58]. It remains to be determined if there are major functional differences between these varying lengths of kisspeptin; some evidence shows that only certain forms will elicit specific responses [59]. Expression of *Kiss1* gene has been observed in many tissues, including the central nervous system, pituitary, testis, small intestine, liver, and pancreas [57, 58]. Studies have also characterized the receptor for kisspeptin as Kiss1r, a member of the galanin family of receptors [60]. All forms of kisspeptin have been shown to equally stimulate Kiss1r [57]. In 2003, the Kisspeptin/Kiss1r system was found to be involved in regulating reproductive function of mammals, with particular relevance to the control of onset of puberty, feedback control of gonadotropin secretion, and generation of the preovulatory surge of LH.

Kisspeptin has also been investigated for its potential role in placental development. Kisspeptin is synthesized by the human placenta [56] and circulating concentrations of kisspeptin increase during pregnancy [61]. Shortly after parturition, plasma concentrations of kisspeptin return to those observed in non-pregnant women.

Bilban et al. [59] observed elevated *Kiss1* gene expression in the trophoblast during the first trimester of pregnancy in woman and suggested a role for kisspeptin in regulating trophoblast invasion into the endometrium. *Kiss1* expression is observed in the oviduct of female rats as well [62]. There, kisspeptin may be important for preventing ectopic pregnancies by inhibiting embryonic implantation in the oviduct. These actions of kisspeptin in the oviduct and placenta are consistent with kisspeptin's role in inhibiting cancer cell invasion and tumor progression [56].

Intracellular Signaling Pathways Induced by Activation of the Kisspeptin-Kiss1r System

It has been suggested that kisspeptin acts solely through Kiss1r as no other receptor has been observed to mediate the effects of kisspeptin in the hypothalamic-pituitary-gonadal (HPG) axis [8]. Kiss1r is coupled with $G_{q/11}$ protein and upon ligand binding causes phospholipase C (PLC) production. PLC triggers phosphatidylinositol 4,5-bisphosphate digestion, generating diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP3) as end products. DAG activates protein kinase C (PKC) while IP3 mobilizes intracellular Ca^{2+} and subsequently alters cellular activity [57]. In GnRH neurons, elevated intracellular Ca^{2+} stimulates neurohormone release via activation of canonical transient receptor potential-like channels and inhibition of K^{+} channels [63]. Blockade of PLC or depletion of intracellular Ca^{2+} stores caused a complete lack of GnRH release due to kisspeptin injections in female rat hypothalamic explants [64].

Kisspeptin binding to Kiss1r has also been shown to activate the MAPK pathway via ERK1/2 phosphorylation [64]. Interestingly, obstruction of ERK 1/2 activation inhibits GnRH release in response to kisspeptin in hypothalamic explants collected from prepubertal rats pretreated with ERK 1/2 inhibitor [64]. The involvement of ERK 1/2 pathway in kisspeptin-induced cellular signaling has been demonstrated in humans as well. A mutation in Kiss1r has been shown to cause prolonged cellular responses to kisspeptin by extending activation of the ERK 1/2 [65]; this mutation was associated with precocious puberty in a young girl. Additional signaling pathways have been proposed as mechanisms of action for the kisspeptin regulation of cell proliferation, migration and apoptosis, and include PI3K/Akt, PKC, and CXCR4 pathways [66]. However, the role of these pathways remains to be fully determined.

As for many G protein-coupled receptors [67], Kiss1r seems to undergo internalization and desensitization after ligand binding. After an initial rise, concentrations of LH decrease with continuous infusion of kisspeptin in the rhesus monkey [67], suggesting desensitization of Kiss1r in GnRH neurons. In contrast, intermittent administration of kisspeptin produces sustained episodic release of LH in the same animal model [68]. In sheep, continuous infusion of kisspeptin for 4 h produced an increase in concentrations of LH in the circulation with a decay curve corresponding to the half-life of the hormone [8]. Although evidence for Kiss1r desensitization also exists in the sheep, low doses of kisspeptin administered continuously to anestrous ewes sustain elevated concentrations of LH for over 24 h [69].

Role of Kisspeptin in the Onset of Puberty

In both humans and mice, disruption of the kisspeptin-Kiss1r pathway impaired normal pubertal development. De Roux et al. [12] and Seminara et al. [13] reported that a homozygous mutation in Kiss1r resulted in hypogonadotropic hypogonadism in humans. This condition was also observed in Kiss1r knockout mice [12, 13]. Characteristics of this condition include decreased gonadal development, diminished production of sex steroids, and failure to obtain puberty. Supplementation with exogenous GnRH restores pubertal development and allows for normal reproductive function in men [70].

The finding that normal functioning of the Kiss1/Kiss1r system is required for puberty to occur in mammals [13] has stimulated large interest in understanding the role of kisspeptin in the regulation of reproductive neuroendocrine function. Kisspeptin receptor is detected in GnRH neurons, suggesting a direct pathway for kisspeptin to regulate GnRH neuronal function [8]. Kisspeptin may also regulate GnRH neurons through a transynaptic pathway by acting on hypothalamic neurons that modulate GnRH activity [71, 72]. Evidence demonstrates that kisspeptin acts primarily at the level of GnRH neurons to stimulate the hypothalamic-gonadotropic axis [8, 11, 73, 74], although direct effects at the pituitary have also been suggested [75-77]. Smith et al. [74] detected Kiss1r mRNA in pituitary tissue of ewes as well as kisspeptin in the hypophyseal portal circulation.

During pubertal development, there is a significant increase in expression of both *Kiss1* and *Kiss1r* genes in the hypothalamus of male and female rats [15]. However, in

monkeys, no increase in *Kiss1r* mRNA expression was seen in males during pubertal development [14]. In both rats and mice, increased GnRH release in response to kisspeptin has been demonstrated during pubertal development [16, 64]. Gonadectomized, prepubertal rats showed increases in hypothalamic *Kiss1* mRNA. The effects of gonadectomy are reversed upon supplementation of gonadal steroids [15]. Immature, undernourished female rats had decreased gonadotropin release and hypothalamic *Kiss1* expression compared to control-fed counterparts [11]. When given repeated injections of kisspeptin, 60% of the undernourished rats showed vaginal opening characteristic of the pubertal stage, and all kisspeptin-treated animals showed robust increases in circulating concentrations of estradiol and gonadotropins [11]. Castellano et al. [11] noticed an increase in *Kiss1r* expression in these malnourished rats and suggested this increased expression was an attempt to counteract the decreased *Kiss1* mRNA. However, it has also been observed that stress-induced suppression of LH secretion in female rats is in part due to down-regulation of hypothalalmic *Kiss1* and *Kiss1r* expression [78]. Repeated injections of kisspeptin given to prepubertal female rats under normal nutritional status induced precocious puberty as determined by early vaginal opening and activation of the gonadotropic axis [79]. These studies further emphasize kisspeptin's role in pubertal development of the gonadotropic axis and suggest an interaction between the regulation of metabolism and kisspeptin neurons.

Kisspeptin's Role in Mediating the Feedback Control of Gonadotropin Secretion by Gonadal Steroids

Feedback effects of estradiol are mainly executed through estrogen receptor (ER)-α. While it is accepted that GnRH neurons do not contain ER-α [80], ovine hypothalamic kisspeptin neurons have been shown to express this receptor [69]. Estrogen receptor-β has been detected on GnRH neurons suggesting a potential direct action of estradiol on GnRH neurons [81]; however evidence indicates that ER-α appears to play a major role in estradiol negative feedback [82]. Placement of estradiol microimplants in the POA and hypothalamus has demonstrated that a major site for the positive feedback effects of estradiol on GnRH release in the ewe is the mediobasal hypothalamus (MBH) [83]. Actions of estradiol at both the POA and MBH seem to be involved in mediating the negative feedback effects of estradiol in the ewe. Since kisspeptin neurons have direct synaptic inputs onto GnRH neurons [84] and kisspeptin neurons contain ER-α [85], it is hypothesized that estradiol effects on GnRH release is mediated by kisspeptin.

The expression of the *Kiss1* gene is regulated by sex steroids. *Kiss1* mRNA increases in the ARC after ovariectomy in the ewe [86] and the number of cells expressing *Kiss1* mRNA increase during the breeding season in the sheep [9]. Studies investigating *Kiss1* mRNA within the POA and hypothalamus suggest that distinct populations of kisspeptin neurons have different roles in regulating reproduction in mammals. Estradiol plays a significant role in the seasonal regulation of reproduction [87]. The effect of estradiol on seasonal changes in reproductive activity in ewes

appears to involve kisspeptin neurons in the arcuate nucleus (ARC), because *Kiss1* mRNA is found to be increased in cyclic ewes compared to non-cyclic ewes [88].

As mentioned earlier in this review, melatonin plays a role in the regulation of reproductive seasonality in sheep [48]; however, it remains to be determined whether kisspeptin neurons are direct targets of melatonin. Furthermore, the proportion of GnRH neurons in close proximity to kisspeptin-containing fibers was greater in the MBH of ovariectomized ewes containing an estradiol implant during the breeding season than during the anestrous season [89]. The number of kisspeptin close contacts per GnRH cell was also greater during the breeding season than the non-breeding season [89]. The differences in *Kiss1* expression and kisspeptin fiber projections were not clear in the POA. Similar counts of kisspeptin cells were seen in both intact and ovariectomized ewes when compared with each other during the breeding and anestrous seasons [88]. In the ovariectomized ewes, treatment with estradiol decreased the number of kisspeptin cells, demonstrating the negative feedback effects of estradiol.

Plasma concentrations of LH increase after injections of kisspeptin in women [90], with the greatest increase during the preovulatory period. Following menopause, a robust increase in GnRH mRNA is observed in humans and non-human primates [91, 92]. A similar increase in *Kiss1* mRNA is observed in the infundibular nucleus of post-menopausal women, indicating that estradiol negative feedback on the *Kiss1* gene is also evident in humans [93].

The positive feedback of estradiol that results in the induction of the preovulatory surge of gonadotropins seems to occur in distinct regions of the hypothalamus,

depending on species. In ewes [83] and monkeys [94, 95], estradiol implants inserted into the MBH induce a surge-like release of LH. In contrast, in the rat, the area stimulated by estradiol encompasses the anteroventral periventricular nucleus (AVPV) [96]. In mice, ovariectomy increases *Kiss1* expression in the ARC, but decreases *Kiss1* expression in the AVPV [10]. Estradiol replacement in ovariectomized mice decreases *Kiss1* gene expression in the ARC, but increases *Kiss1* mRNA in the AVPV [10]. Further evidence for the role of kisspeptin mediating estradiol positive feedback in rodent species is provided by the demonstration that *Kiss1* mRNA in the AVPV is dramatically increased in mice on the evening of proestrus at the time of the preovulatory surge of LH. This observation indicates that the population of kisspeptin cells in the AVPV is a target for estradiol's stimulation of the preovulatory surge of LH in rodents.

Progesterone also exerts positive and negative effects on secretion of gonadotropins. In estradiol-primed female mice and rats, progesterone stimulates a surge of release of LH [97]. It has been demonstrated in the female mouse that kisspeptin neurons in the rostral periventricular area of the third ventricle contain the progesterone receptor (PR) [98], suggesting that progesterone can directly affect kisspeptin neuronal function. In the ewe, the majority of kisspeptin neurons in the ARC contain PR [9]. However, unlike in the mouse, progesterone blocks the estradiol-induced surge of GnRH/LH release [99]. Therefore, the role of progesterone in the regulation of gonadotropin release is unclear in the sheep. Stimulation of the LH surge is a multi-input process and may involve transmission of information through various

cell types within the POA and hypothalamus to the GnRH neuron [100]. Nevertheless, pathways involving kisspeptin neurons are assumed to be greatly involved because injections of a monoclonal antibody to kisspeptin in the POA abolishes cyclicity and the estrogen-induced LH surge in female rats [101, 102].

Cortisol, a major adrenal hormone released into the bloodstream in response to stress, delays the onset of the preovulatory LH surge in ewes [103, 104]. The mechanisms of cortisol's negative actions upon the hypothalamic-pituitary-gonadal axis remain to be determined, but it seems to involve actions through the type II glucocorticoid receptor, likely at the level of the pituitary [105]. A recent study performed by Iwasa et al. [106] demonstrated that *Kiss1* mRNA in the hypothalamus decreased in female rats injected with lipopolysaccharide, a model used to induce activation of the stress axis and immune responses that inhibit gonadotropin release. Injections of exogenous kisspeptin stimulate release of LH in rats injected with lipopolysaccharide in a manner similar to that exhibited in control-treated rats [106]. Therefore, immune/stress responses may also affect reproductive endocrine function by inhibiting kisspeptin synthesis and release.

Kisspeptin in the Male

Stimulation of the reproductive endocrine axis by kisspeptin in males has been demonstrated in various mammalian species. Dhillo et al. [94] investigated the effects of kisspeptin in man and observed that kisspeptin increases circulating concentrations of LH, FSH, and testosterone. A single injection of kisspeptin increases concentrations of

LH in juvenile agonadal monkeys [14] as well as in adult mice [107] and rats [15]. Moreover, orchidectomy in rats increases hypothalamic *Kiss1* mRNA [15] and in male orchidectomized rats fitted with testosterone implants, *Kiss1* mRNA levels are similar to intact males. In the *Kiss1r* -/- male mouse, testicular size and concentrations of testosterone in circulation are reduced, and spermatogenesis impaired [13]. In this knockout model, the release of GnRH seems to be impaired because synthesis of GnRH remains unaffected and treatment with exogenous GnRH induces release of LH and follicle stimulating hormone (FSH) from the pituitary [13].

Male seasonal breeders exhibit decreased reproductive activity during the non-breeding season. Hamsters typically restrict reproductive activity to spring and summer months. In the male hamster, decreased activation of the hypothalamic-pituitary-gonadal axis leads to lower circulating concentrations of testosterone in the off season. Greives et al. [108] observed that the number of immunoreactive kisspeptin cells in Siberian hamsters is reduced in the AVPV and increased in the ARC during the non-breeding season, and suggested that changes in kisspeptin cell numbers are due to photoperiodic and gonadal influences.

Metabolic Regulation of Kisspeptin

In addition to being regulated by steroid hormones, kisspeptin neurons are regulated by metabolic factors as well. Short-term (72 h) fasting in prepubertal male and female rats decreases *Kiss1* mRNA in the hypothalamus and causes a slight increase in hypothalamic *Kiss1r* mRNA [11]. Similar decreases in hypothalamic *Kiss1* mRNA are

observed in food-restricted adult female rats [109] and male mice [110]; however, *Kiss1r* mRNA decreases in the adult male mice [109, 110], unlike in the prepubertal rats. Differences in these studies can be due to differences in age, species, and duration of fasting. It is also possible that *Kiss1r* mRNA initially decreases in response to fasting, but with the concurrent decrease in *Kiss1* expression, *Kiss1r* mRNA compensates and returns to pre-fasting levels due to lack of kisspeptin stimulation.

Smith et al. [111] reported that the long form of the leptin receptor is present in kisspeptin neurons, indicating a direct effect of leptin upon kisspeptin neurons. Indirect effects through leptin-sensitive neurons in the ARC are also suggested [111]. The role of leptin on the regulation of kisspeptin neurons was demonstrated by Castellano et al. [112] in leptin-deficient, hypogonadotropic diabetic male *ob/ob* mice. Continuous infusions of leptin partially restored *Kiss1* mRNA, LH, and testosterone, as well as prostate and testes weights. Therefore, it is proposed that leptin is required for normal function of kisspeptin neurons and its downstream effects on events leading to the onset of puberty.

CHAPTER III

KISS1 GENE EXPRESSION IN THE PREOPTIC AREA AND HYPOTHALAMUS DURING MATURATION OF THE REPRODUCTIVE NEUROENDOCRINE AXIS IN EWE LAMBS

Introduction

Pubertal maturation of the reproductive neuroendocrine axis in mammalian species is characterized by an increase in the frequency of episodic release of luteinizing hormone (LH) [3, 26, 113]. The increase in frequency of LH release occurs as a result of elevated gonadotropin-releasing hormone (GnRH) release [114] following the juvenile hiatus in gonadotropin secretion [4]. Ultimately, the pubertal increase in GnRH neuronal activity is supported by, at least in part, augmented excitatory stimuli that promote GnRH neurosecretion [115, 116]. Kisspeptin, a product of the *Kiss1* gene, is a potent stimulator of GnRH release [8] and may play a vital role in pubertal activation of the reproductive neuroendocrine system [8]. The gene encoding kisspeptin receptor, *Kiss1r,* is expressed in GnRH neurons [8] and mutations in this gene result in hypogonadotropic hypogonadism and failure to attain normal puberty [13].

Neurons containing *Kiss1* mRNA and peptide are located in the preoptic (POA) and periventricular (PeV) areas (anteroventroperiventricular area, AVPV, in rodents), and in the arcuate nucleus (ARC) [9, 107]. Expression of *Kiss1* gene increases with puberty in female mice [16] and rats [15]. Similarly, *Kiss1* mRNA increases from early-pubertal to mid-pubertal development in female monkeys [14]. However, whether changes in *Kiss1* expression occur in distinct neuronal populations during pubertal

transition to elevated episodic release of LH is unclear. Importantly, populations of kisspeptin neurons are differentially regulated by estradiol, with estradiol stimulating *Kiss1* expression in the preoptic area [10], but inhibiting *Kiss1* expression in the ARC nucleus [9, 10]. Therefore, changes in negative feedback effects of estradiol regulating episodic release of LH during pubertal transition [24, 117] may involve regulation of distinct populations of kisspeptin neurons. In the study reported herein, we tested the hypothesis that *Kiss1* gene expression in the POA and hypothalamus increases in ewe lambs transitioning toward elevated pulsatile release of LH characteristic of pubertal states. Specifically, changes in *Kiss1* mRNA in the POA/PeV and ARC were determined in ewe lambs exhibiting prepubertal, peripubertal and pubertal patterns of episodic LH release.

Materials and Methods

Experiments were conducted at the Texas A&M University Physiology Field Laboratory and O.D. Butler Animal Science Teaching and Research Complex, College Station, Texas. The Institutional Agricultural Animal Care and Use Committee of the Texas A&M University system approved all procedures used in these studies.

Animals and experimental procedures

Eighteen spring-born ewe lambs, weaned at 8 wks of age, were used for this study. Ewe lambs were ovariectomized between 15 and 20 wks of age and an estradiol implant designed to produce circulating concentrations of estradiol of approximately 2

pg/ml was inserted subcutaneously at the time of ovariectomy. Lambs were fed *ad libitum* a complete mixed diet containing 16% crude protein and 2.8 MCal/Kg of metabolizable energy formulated to promote weight gain of approximately 0.2 to 0.25 kg/d. Mineral mixture and water were available *ad libitum*. Ewe lambs were stratified by day of birth and assigned randomly to one of three groups (n=6/group). Ewe lambs in each group were euthanized at 25 wk of age (Group 1; prepubertal), 30 wk of age (Group 2; peripubertal), and 35 wk of age (Group 3; pubertal) with an overdose of sodium pentobarbital (Beuthanasisa-D Special, Schering-Plough, Union, NJ). After confirmation of death, based on absence of audible heartbeat and visual signs of respiration, animals were decapitated and the brain removed from the skull. A block of tissue containing the septum, POA and hypothalamus was collected and snap frozen in liquid nitrogen vapor. Tissue blocks were stored at -80^{o} C until processing.

On the day before euthanasia, blood samples were collected every 10 min for 12 h via a catheter (polypropylene tubing 0.86mm i.d. x 1.52mm o.d.) inserted into the external jugular vein. During blood sampling, ewe lambs were maintained in small pens and secured loosely with a halter. Catheters were inserted into the jugular vein on the day before blood sampling began and all ewe lambs were acclimated to experimental conditions for 5 d before blood sampling. To minimize isolation stress, another sheep was maintained in an adjacent pen at all times during sampling. Extension tubing was used to allow remote sampling from the jugular catheter. Blood samples (5ml) were placed in tubes containing 50µl of a solution of heparin (3,000U/ml) and 5% EDTA. Tubes containing blood samples were placed immediately on ice and centrifuged at 2200

x g for 20 min at 4° C within 3 h of collection. Plasma was collected and stored at -20° C until processed for determining concentrations of LH. Jugular catheters were flushed with sterile physiological saline solution after every sampling and with heparinized saline every 30 min to prevent clotting of the catheter.

Tissue processing and in situ hybridization

Frozen tissue blocks were cut in coronal sections of 20 µm using a cryostat (Leica CM 1900). Sections were thaw-mounted onto slides and frozen. Slides were then stored at -80° C until processed for in situ hybridization.

For the detection of *Kiss1* mRNA, antisense radiolabeled cRNA probes were generated by in vitro transcription of a linearized plasmid containing a partial sequence of the ovine *Kiss1* cDNA (GenBank accession number DQ059506) and used for *in vitro* hybridization histochemistry. Sense probes were generated from the same plasmid and used as controls. The in vitro transcription reaction contained transcription buffer (Promega, Madison, WI, USA), 0.5 mM ribonucleotide triphosphates (rATP, rGTP, rCTP), 10 µM rUTP, 1 µg DNA template, 10 µM dithiothreitol (DTT), 5 µM RNase inhibitor (RNasin, Promega), 20 U of T7 or SP6 RNA polymerases (Promega), and 50 µCi ^{35}S-UTP (MP Biomedicals, Solon, OH, USA). Reaction was incubated for 4 h at 37° C. DNase-I (Sigma, St. Louis, MO, USA) was added to the reaction to degrade the DNA template followed by an additional incubation at 37° C for 15 min. The reaction was stopped with 0.5 M EDTA, unincorporated nucleotides were removed using a

NucAway Spin Column (AB Ambion, Austin, TX, USA), and 10 µg tRNA was added to probe solution.

Slides containing tissue sections were removed from the freezer, air dried for 2 min and fixed in fresh 4% paraformaldehyde diluted in 0.1M PB (pH 7.2) for 15 min at room temperature. Sections were then washed in 0.1M PB twice for 5 min each and placed in a Proteinase K solution (0.5 µg/ml Proteinase K [Invitrogen, Carlsbad, CA], 5.0 mM EDTA, 50 mM Tris-HCl) for 30 min at 37^{o} C. Following this incubation, sections were rinsed in 0.1M PB and returned to the 4% paraformaldehyde solution for 5 min, followed by rinsing in 0.1M PB, rinsed in water twice, and dipped in 0.1 M triethanolamine (TEA) and TEA containing acetic anhydride for 10 min with constant stirring at room temperature. This was followed by washing twice in 2X saline-sodium citrate (SSC), dehydration through graded ethanol (70%, 95%, and 100%), delipidation in chloroform for 5 min, washing in 100% and 95% ethanol, respectively, for 3 min each, and air drying. Sections were then hybridized with sense or antisense radiolabeled cRNA probes for *Kiss1*. Before hybridization, probes were diluted in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM $NaPO_4$, Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml of yeast tRNA, and 100mM DTT) and denatured at 70^{o} C for 10 min. Hybridization solution was applied to tissue sections and a parafilm coverslip placed on each slide. Sections were hybridized overnight at 55^{o} C in a humidified chamber.

After overnight incubation, the coverslips were removed and sections washed once in 5X SSC containing 10 mM β mercapto-ethanol (βME) for 30 min at 55^{o} C

followed by TEN (10 mM Tris-HCL, pH 8; 5 mM EDTA, pH 8; 0.5 M NaCl) buffer for 10 min three times at 37° C. Sections were then treated with Ribonuclease (RNase) in TEN buffer for 30 min at 37° C followed by another wash in TEN for 30 min at 37° C. This was followed by washing twice in 5X SSC containing 10 mM βME for 30 min and 15 min at 55° C, once in 0.1X SSC containing 10mM βME for 15 min at 55° C, and once in 0.1X SSC for 15 min at room temperature. Sections were then dehydrated through a series of ethanol solutions (70% ethanol + 0.3 M ammonium acetate twice for 5 min, 95% ethanol + 0.3 M ammonium acetate once for 3 min, and 100% ethanol twice for 2 min) and air dried for 1 to 3 h at 37° C. Sections were dipped in photographic NBT2 emulsion, dried, and exposed in the dark for 10 d at 4° C. Slides were developed in D-19 developer and sections counterstained with cresyl violet, dehydrated, and covered with a glass coverslip using DPX.

Analysis

Hybridized slides were coded and analyses of tissue sections were performed by an observer unaware of experimental group composition. Every tenth section (200 µm apart) through the POA and hypothalamus was analyzed for each ewe. Cells containing *Kiss1* mRNA were identified using dark- and bright-field microscope (Nikon 80i Eclipse, Nikon Inc., Melville, NY, USA) on the basis of area covered by silver grains (at least five times above background levels). Images of labeled material were captured using a digital camera (DS-Qi1; Nikon) attached to the microscope. Images were

imported into Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and were not altered in any way except for adjustments of brightness and contrast.

Location and number of Kiss1 cells. Location and number of cells expressing the *Kiss1* gene were determined in three sections through the preoptic area (including one section at the level of the organum vasculosum of the lamina terminalis), two through the PeV and six through middle ARC. Sections were selected from each ewe lamb to represent comparable levels through the POA and hypothalamus of all animals.

Kiss1 expression per cell. For each area examined, bright-field photomicrographs were captured at 40X magnification (Nikon 80i Eclipse) from 30 *Kiss1* mRNA-containing cells in at least 2 representative sections in both the POA/PeV and ARC of each ewe to estimate area covered by silver grains. The NIS-Elements software (Nikon) was used to determine the area covered by silver grains. Briefly, a region of interest (ROI) of 20 μm in diameter was placed over the cell. Three additional ROIs of the same size were created and placed randomly throughout the image to determine background deposits of silver grains. A threshold signal was established and applied to all images before the number of pixels was determined in standardized ROI. The number of pixels within each ROI was recorded to represent the area covered by silver grains per cell and data were used for analysis to estimate *Kiss1* expression per cell.

Hormone assays

Concentrations of LH in plasma samples were determined by a double antibody radioimmunoassay (RIA) using rabbit anti-ovine LH (AFP192279; National Hormone and Pituitary Program, NHPP, Torrance, CA) and ovine LH (AFP8614B; NHPP) as the labeled tracer and reference preparation. Briefly, 200 µl of reference standard preparation or plasma were added to polypropylene tubes containing 300 µl of a 1% egg white-phosphate buffered saline solution (1% EW-PBS). Two hundred µl of a solution containing ovine LH antiserum (1:1,250,000 dilution in PBS + 0.05 M EDTA + 1:400 NRS) were added to assay tubes and mixed by vortex. After one to two hours incubation at room temperature, 100 µl of a solution containing labeled tracer (20,000 cpm in 0.1% EW-PBS) were added to all assay tubes and mixed by vortex. Following 24 h incubation at 4^{o} C, 200 µl of a solution containing goat-anti-rabbit gamma globulin (Equitech-Bio Inc., Kerrville, TX) were added to each tube. The tubes were again vortexed and set to incubate at 4^{o} C for 48 to 72 hrs. After incubation, 3 ml cold PBS (0.01 M; pH 7.0) was added to each tube on a per spin basis. The tubes were then centrifuged at 2200 X g for 1 hr at 4^{o} C. The supernatant was then decanted and the tubes counted in a gamma counter for 1 min. Intraassay and interassay coefficients of variation averaged 9.4% and 15.7%, respectively.

Statistical analysis

Frequency and amplitude of LH pulses were determined using a pulse-detection algorithm (Pulsefit 1.2) [118]. Body weight, hormone data, number of cells expressing

Kiss1 mRNA and the area covered by silver grains/cell were analyzed by analysis of variance (ANOVA) using the PROC GLM procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Group (25, 30, and 35 wks of age) was used as the main sources of variation. When significant differences were detected using ANOVA, the least squares means were used to compare means between groups.

Because the frequency of LH pulses was highly variable among animals in the 30-wk of age group, regression analysis was performed to investigate whether number of *Kiss1*-expressing cells and the area covered by silver grains per cell changed relative to increases in frequency of episodic release of LH. Subsequently, ewe lambs were reallocated in two groups based on the number of LH pulses as follows: 1) Low Frequency (0 to 5 pulses/12 h; n=9), which included all 25-wk old ewes and three of six 30-wk-old ewes; and 2) High Frequency (6 or more pulses/12 h; n=9), which included all 35-wk old ewes and the remaining three 30-wk-old ewes. Mean number of *Kiss1* expressing cells and area covered by silver grains per cell was then reanalyzed for both the POA/PEV and ARC.

Results

Body weight of ewe lambs increased with age at expected rates during the experiment. Mean body weight of 35-wk-old ewe lambs (55.2 ± 2.4 kg) was greater ($P < 0.01$) than 30- (44.2 ± 2.9 kg) and 25- (38.2 ± 3.0) wk-old ewes. Similarly, mean circulating concentrations of LH and frequency of LH pulses increased with age and 35-wk-old ewes had a greater ($P < 0.01$) frequency of LH pulses than 30- and 25-wk-old ewes (Table 3.1). Mean amplitude of LH pulses did not differ among groups (Table

3.1). Patterns of LH release during the 12 h sampling period in two representative ewe lambs in each age group are depicted in Figure 3.1.

Table 3.1. Mean circulating concentrations of LH, and mean frequency and amplitude of LH pulses in 25-, 30-, and 35-wk-old ewe lambs.

	Age (wk)		
	25	30	35
Concentrations (ng/ml)	1.0 ± 0.02^{a}	2.3 ± 0.08^{b}	3.5 ± 0.07^{c}
Frequency (pulses/12 h)	1.7 ± 0.8^{d}	4.5 ± 1.8^{d}	10.5 ± 1.3^{e}
Amplitude (ng/ml)	0.6 ± 0.3	1.9 ± 0.6	1.9 ± 0.3

[a, b, c] Differ ($P < 0.001$); [d, e] Differ ($P < 0.01$).

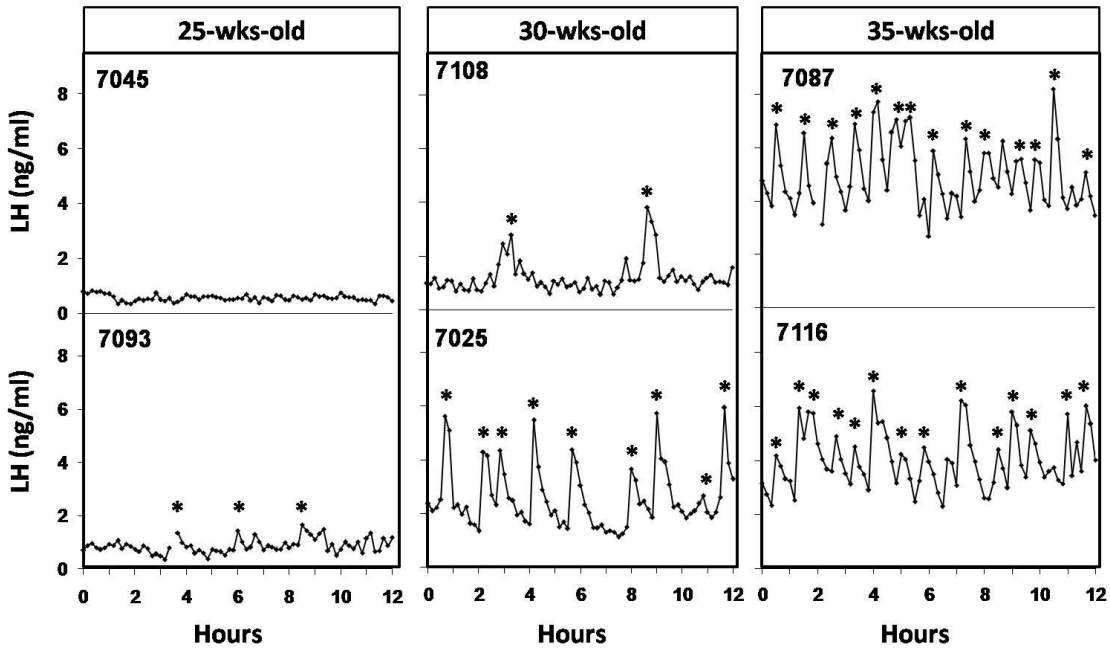

FIG. 3.1. Circulating concentrations of LH in representative ewe lambs at 25, 30 and 35 wk of age on the day before brain tissue collection. Detected pulses are depicted with asterisks. Frequency of LH pulses increased with age in growing ewe lambs.

In one 25-wk-old ewe lamb, tissue from the rostral aspect of the POA was missed during sectioning, resulting in lack of comparable POA sections for analysis. Therefore, data used for analyses of number of *Kiss1*-mRNA containing cells and area covered by silver grains per cell in the POA/PeV of 25-wk-old lambs included sections from 5 animals. Hybridization of tissue sections with antisense probe to *Kiss1* mRNA labeled an abundant number of cells in the POA, PeV (Fig. 3.2) and ARC (Fig. 3.3) regions of ewes in all age groups. In the POA/PeV, the number of *Kiss1*-expressing cells was less ($P < 0.04$) in 25-wk-old ewe lambs than in 30- and 35-wk-old ewes (Table 3.2). However, number of *Kiss1*-expressing cells in the POA/PeV did not change relative to

increases in the frequency of LH pulses (Fig. 3.4). In the ARC, no differences in the number of *Kiss1*-containing mRNA cells among the three age groups were observed, although a trend toward increased number of cells with age seems to exist (Table 3.2). Importantly, a linear increase ($P < 0.014$) in the number of *Kiss1*-mRNA containing cells relative to increases in the frequency of LH pulses were observed in the ARC (Fig. 3.5). No differences in area covered by silver grains per cell were observed among age groups in none of the kisspeptin neuron populations investigated (Table 3.2).

Because the number of cells expressing the *Kiss1* gene in the ARC increased with increases in the frequency of episodic LH release (Fig. 3.5), ewe lambs were reallocated to two groups based on LH pulsatility to low (0 to 5 pulses/12 h) and high (6 or more pulses/12 h) frequency groups. All 25-wk-old and 3/6 of 30-wk-old ewe lambs were allocated to Low Frequency group (n=9). All 35-wk-old and 3/6 30-wk-old lambs were allocated to High Frequency group (n=9). The number of *Kiss1*-cells in the ARC was greater ($P < 0.02$) in ewe lambs exhibiting a high frequency of LH pulses than in ewe lambs exhibiting a low frequency of LH pulses (Fig. 3.6). Area covered by silver grains per cell in the ARC did not differ between groups.

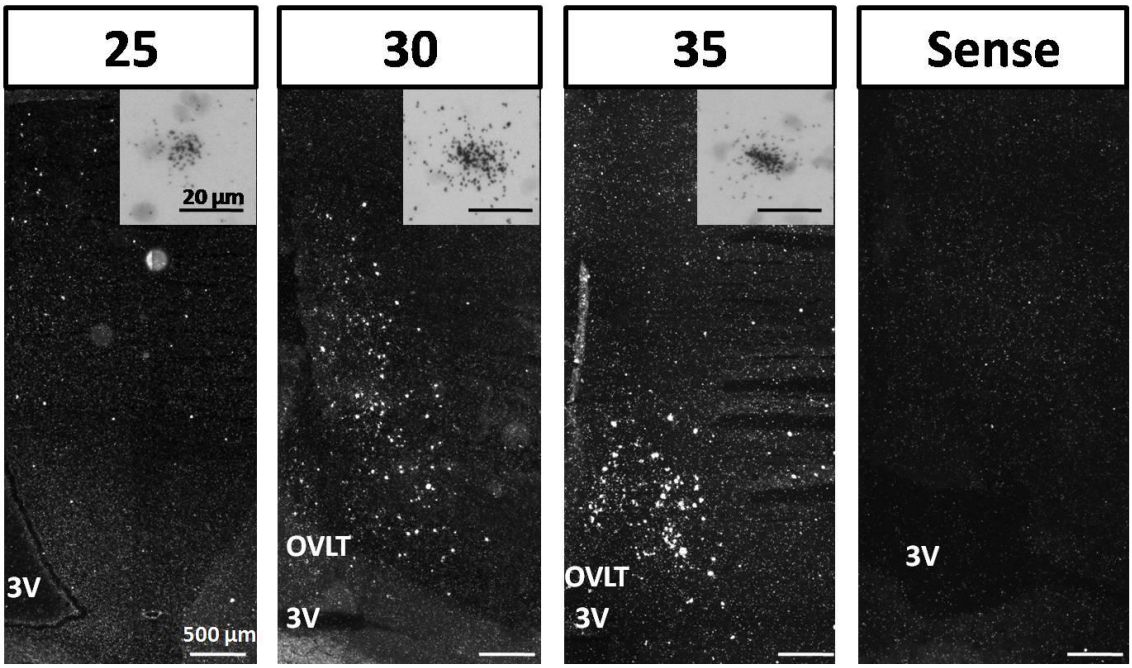

FIG. 3.2. Photomicrographs of sections processed for in situ hybridization depicting cells containing *Kiss1* mRNA in the POA of representative 25- (A), 30- (B) and 35-wk-old (C) ewe lambs. In low-power, dark-field photomicrographs (A-C), *Kiss1*-expressing cells are observed as white clusters of silver grains in sections hybridized with antisense probe for ovine *Kiss1*. No cells are detected in sections hybridized with sense probe (D). In high-power, bright-field photomicrographs, *Kiss1*-expressing cells are observed as black clusters of silver grains accumulating over Cresyl-violet counterstained cells (a-c). Scale bars: (A-D), 500 µm; (a-c), 20 µm. OVLT, organum vasculosum of the lamina terminalis.

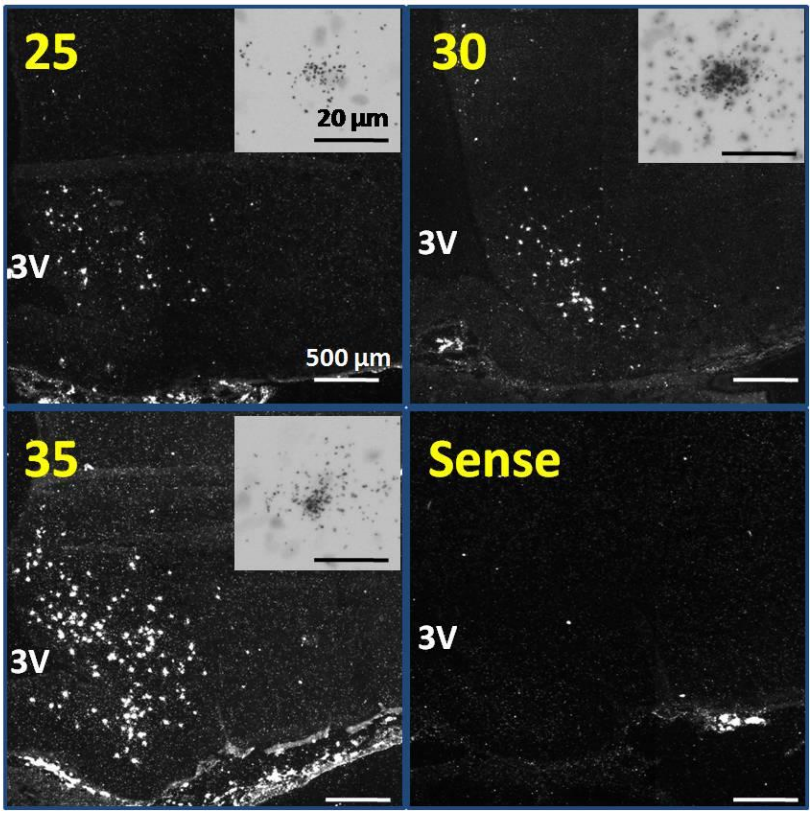

FIG. 3.3. Photomicrographs of sections processed for in situ hybridization depicting cells containing *Kiss1* mRNA in the middle ARC of representative 25- (A), 30- (B) and 35-wk-old (C) ewe lambs. In low-power, dark-field photomicrographs (A-C), *Kiss1*-expressing cells are observed as white clusters of silver grains in sections hybridized with antisense probe for ovine *Kiss1*. No cells are detected in sections hybridized with sense probe (D). In high-power, bright-field photomicrographs, *Kiss1*-expressing cells are observed as black clusters of silver grains accumulating over Cresyl-violet counterstained cells (a-c). Scale bars: (A-D), 500 µm; (a-c), 20 µm. 3V, third ventricle.

Table 3.2. Mean number of *Kiss1*-expressing cells in the POA/PeV and ARC, and area covered by silver grains per cell in 25-, 30- and 35-wk-old ewe lambs.

		Age (wk)		
		25	30	35
Number of cells	POA/PEV	123.5 ± 21.7[a]	194.0 ± 20.3[b]	189.2 ± 15.2[b]
	ARC	118.2 ± 11.8	176.3 ± 53.6	225.5 ± 64.1
Expression per cell (pixels)	POA/PEV	4471 ± 539	4879 ± 588	4779 ± 327
	ARC	4818 ± 404	5384 ± 257	5527 ± 552

[a, b] Differ ($P < 0.04$).

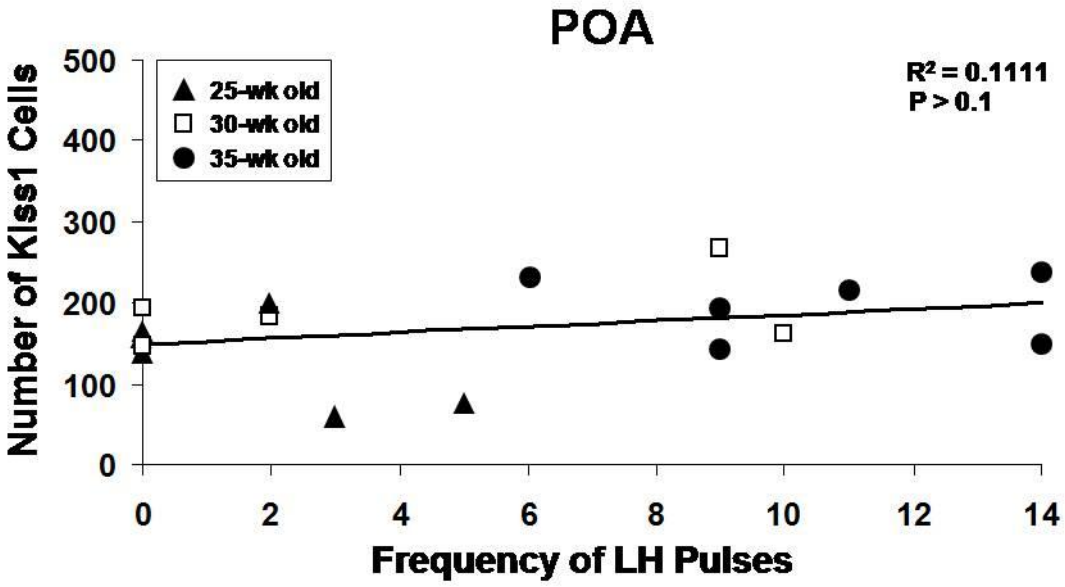

FIG. 3.4. Linear regression of the number of *Kiss1*-expressing cells in the POA/PeV relative to frequency of LH pulses in 25-, 30-, and 35-wk-old, ovariectomized, estradiol-replaced ewe lambs. Number of *Kiss1* cells in the POA/PeV did not differ relative to changes in frequency of LH pulses.

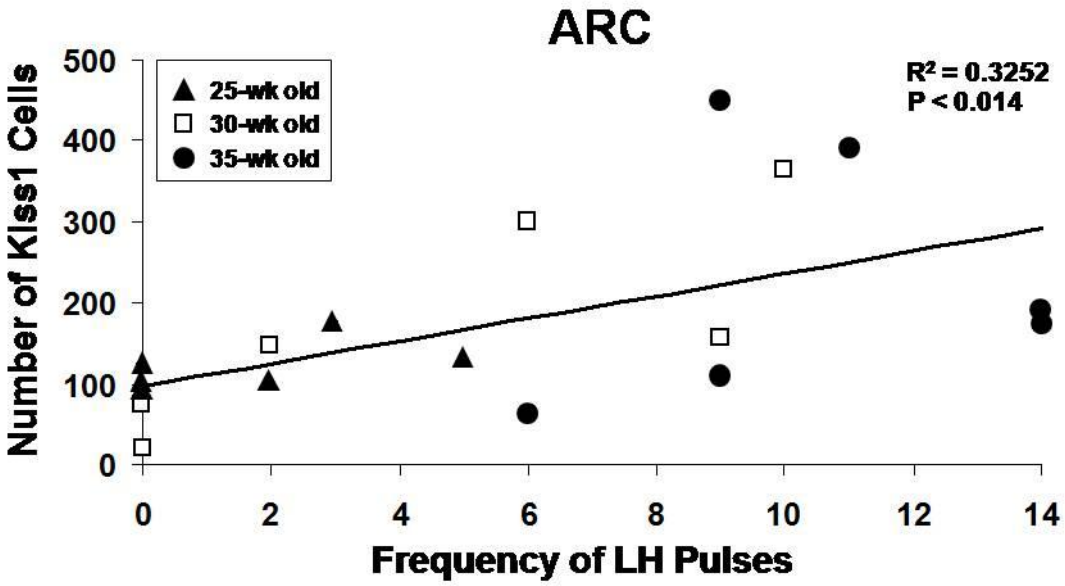

FIG. 3.5. Linear regression of the number of *Kiss1*-expressing cells in the ARC relative to frequency of LH pulses in 25-, 30-, and 35-wk-old, ovariectomized, estradiol-replaced ewe lambs. Number of *Kiss1* cells in the ARC increases ($P < 0.014$) relative to increases in frequency of LH pulses.

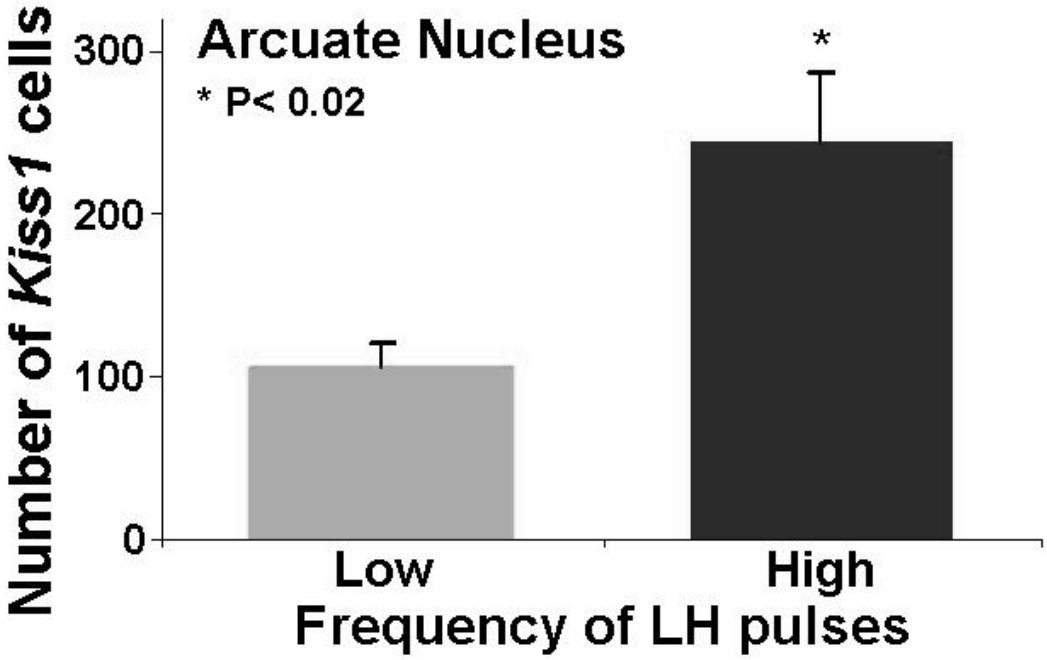

FIG. 3.6. Mean (± SEM) number of *Kiss1* mRNA-containing cells in the ARC of 25 to 35 wk-old ewe lambs exhibiting low (1.3 ± 0.6 pulses/12 h) and high (9.8 ± 1 pulses/12 h) frequency of LH pulses. *Kiss1*-expressing cells in the ARC increase ($P < 0.02$) with heightened frequency of LH release during maturation of the reproductive neuroendocrine axis.

Discussion

The period of pubertal maturation is a time of numerous physiological changes, including the increase in the frequency of LH pulses that support enhanced gonadal steroidogenesis, gametogenesis and first ovulation in females. Our study demonstrates that in ewe lambs, the increase in the frequency of LH pulses is accompanied by an increased number of *Kiss1*-expressing cells within the ARC. This is in contrast to an increase in number of *Kiss1*-expressing cells in the POA/PeV at earlier stages of juvenile

development that are unrelated to changes in the frequency of episodic LH release. These observations expand the current understanding of the role of the kisspeptin system in the process of pubertal development in mammals [13, 18, 119], and demonstrate that an increase in *Kiss1* expression in the ARC occurs at a time conducive for pubertal onset.

Kisspeptin is critical for the onset of puberty and mutations in the *Kiss1* gene [120] or in the kisspeptin receptor [13] impair normal pubertal development. The role of kisspeptin in regulating puberty seems to involve stimulation of gonadotropin release via direct actions on GnRH neurons. The kisspeptin receptor is present in GnRH neurons [13], and kisspeptin enhances GnRH neuronal activity [71] and increases GnRH release [8]. Therefore, increased kisspeptin-stimulation of GnRH neurons at the time of pubertal transition toward patterns of LH release required for maturation of gonadal function may be essential for establishment of normal reproductive capacity.

Increased expression of *Kiss1* gene during pubertal development has been reported in mice [16], rats [121] and monkeys [14], suggesting that activation of *Kiss1* expression may support pubertal onset of heightened GnRH release. Overall increase in *Kiss1* expression in the ARC is observed from the juvenile to adulthood in ovariectomized, estradiol-treated rats [121], and this increase is associated with elevations in the frequency of LH release. Although to a lesser extent, a similar relative increase in *Kiss1* expression was observed in the AVPV [121]. However, no changes in *Kiss1* expression occurred during this developmental period in ovariectomized rats not treated with estradiol. This suggests a role for estradiol in the regulation of *Kiss1*

expression during development. In contrast, in our studies using the ovariectomized, estradiol-replaced ewe lamb, changes in *Kiss1* expression associated with increases in frequency of LH release were observed only in the ARC. Moreover, these changes were associated with increases in the number of *Kiss1*-expressing cells, and not with the level of expression per cell. Therefore, in ewe lambs, the developmental decrease in estradiol negative feedback seems to be associated with activation (or disinhibition) of expression of the *Kiss1* gene in ARC neurons.

The pubertal increase in the frequency of LH release has been associated with diminished sensitivity to estradiol negative feedback in various mammalian species [24, 26, 117, 122]. In sheep, concentrations of estradiol that are inhibitory earlier during the juvenile period no longer inhibit LH release at later stages of development [24]. In absence of estradiol treatment, ovariectomized ewe lambs exhibit patterns of LH release similar to those of mature ewes. As expected in the current studies, ovariectomized, estradiol-replaced 25-wk-old ewes exhibited a low frequency of LH release, indicating heighted sensitivity to estradiol negative feedback. As ewe lambs matured, diminished estradiol negative feedback could be observed by increased frequency of LH release in 35-wk-old ewes. The mechanisms by which estradiol inhibits episodic release of LH are unclear, but may involve regulation of kisspeptin synthesis. Expression of the *Kiss1* gene in the ARC is increased in ovariectomized, mature ewes during the breeding season [9]. Estradiol replacement in ovariectomized ewes inhibits *Kiss1* expression in the ARC to levels similar to intact ewes [9]. Likewise, the number of immunoreactive kisspeptin cells in the ARC is reduced in ovariectomized, estradiol-treated ewes [89]. Thus, taking

into account that kisspeptin has potent stimulatory effects on LH release [8], the inhibitory effects of estradiol on kisspeptin synthesis in the ARC are conducive to decreased stimulation of LH release in response to estradiol negative feedback. However, during states of diminished sensitivity to estradiol negative feedback, the expression of *Kiss1* in the ARC increases concurrent to increases in frequency of episodic LH release. This effect was observed in the current studies during peripubertal transition in the ewe lamb.

The effects of low concentrations of estradiol on kisspeptin expression in the POA of sheep are less clear. Estradiol replacement in ovariectomized ewes does not cause consistent changes in *Kiss1* expression in the POA during the breeding season [9, 89]. However, the number of kisspeptin-immunoreactive cells in the POA is increased in ovariectomized ewes treated with estradiol [89]. Nevertheless, *Kiss1* expression increases in the POA during late follicular phase in ewes, a time during the estrous cycle in which elevated concentrations of estradiol induce the preovulatory surge of GnRH/LH [123]. Although estradiol stimulatory feedback was not investigated in the current studies, it is interesting to note that the expression of *Kiss1* increased in ewe lambs from 25 to 30 wks of age. The ability to respond to estradiol positive feedback develops early during the juvenile period, but a full LH surge in response to estradiol is established only later during the prepubertal stage [22]. Therefore, it is possible that developmental patterns of *Kiss1* expression in the POA in the sheep are less dependent on estradiol negative feedback. In the ewe, only approximately 50 % of the kisspeptin cells in the POA contain estrogen receptor alpha [124]. However, kisspeptin cells of the POA may

be involved in the establishment of the GnRH/LH surge in response to stimulatory effects of estradiol [125, 126]. In rodents, estradiol increases *Kiss1* mRNA in the AVPV [10], and *Kiss1* expression is upregulated in the afternoon of proestrus in mice, a time consistent to the GnRH/LH surge [127]. Nevertheless, elevated estradiol seems to also activate kisspeptin cells in the ARC. Expression of *Kiss1* gene, the number of kisspeptin immunoreactive cells and the proportion of kisspeptin cells expressing Fos, are increased in the ARC during late follicular phase [125].

Developmental changes in the ability of kisspeptin to stimulate GnRH neuronal activity seem to exist. In mice, larger doses of kisspeptin are required to effectively stimulate depolarization of GnRH neurons in adult compared to juvenile animals [12]. This observation suggests that there is a developmental progression in the responsiveness of GnRH neurons to kisspeptin. Although the mechanisms of this functional change are not clear, they may involve increased expression of kisspeptin receptor in GnRH neurons during juvenile development or enhancement of kisspeptin-induced signaling within GnRH neurons. The latter is more likely because no difference in the number of kisspeptin receptors in GnRH neurons is observed between adult and juvenile mice [12].

In sheep, ARC kisspeptin neurons do not seem to project to POA GnRH neurons in considerable numbers [128], but project to GnRH neurons in the mediobasal hypothalamus [129]. Pulsatile release of LH has been associated with activation of GnRH neurons in the mediobasal hypothalamus in ewes [130]. Therefore, it is conceivable that kisspeptin neurons in the ARC are involved with generation of GnRH

pulses by acting on GnRH neuron cell bodies in the mediobasal hypothalamus. Regulation of pulsatile release of GnRH by kisspeptin may also occur by actions at the level of the median eminence. Release of GnRH is stimulated by kisspeptin infusion in the median eminence of monkeys [131]. In addition, kisspeptin and GnRH fibers intermingle in the median eminence [86], and many of these kisspeptin fibers seem to originate from the ARC [86]. The recent studies demonstrating that a kisspeptin antagonist injected in the ARC of rats inhibits pulsatile LH release [132] strengthen the implication of ARC kisspeptin neurons in the generation of GnRH pulses. These observations are in accordance to our findings in the prepubertal ewe and support the notion that activation of kisspeptin neurons in the ARC are involved in the pubertal acceleration of pulsatile release of LH.

In summary, greater numbers of cells containing *Kiss1* mRNA in the ARC are observed in ewe lambs exhibiting increased frequency of LH pulses. This suggests a role for ARC kisspeptin neurons during maturation of the reproductive neuroendocrine axis in sheep. Although unrelated to changes in LH pulsatility, increases in *Kiss1* expression in the POA also occur during juvenile development in the ewe lamb and may be involved in the development of the surge mode of GnRH release.

CHAPTER IV

KISSPEPTIN ACTIVATES THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN PREPUBERTAL EWE LAMBS

Introduction

Maturation of the reproductive neuroendocrine axis in mammalian species is characterized by an increase in the frequency of episodic release of luteinizing hormone (LH) [1-3] in response to pulsatile gonadotropin-releasing hormone (GnRH) from hypothalamic neurons [114]. Sustained elevation of circulating concentrations of LH supports the final stages of follicular development, steroidogenesis and ultimately, ovulation [18]. Although pubertal activation of pulsatile GnRH release has been characterized [20, 133] the mechanisms leading to increased frequency of GnRH release during the peripubertal period remain unclear. Kisspeptin, a peptide with potent stimulatory effects on the release of GnRH and LH [8, 134-138], has been proposed to play a critical role. Kisspeptin and its receptor (Kiss1r) are essential for normal pubertal development [12, 13, 139, 140]. Administration of kisspeptin to prepubertal rats advances the time of vaginal opening [79], suggesting that kisspeptin can hasten maturation of reproductive function. Moreover, kisspeptin prevents undernutrition-induced delayed puberty in rats [11].

The stimulation of reproductive endocrine function by kisspeptin in prepubertal animals is believed to occur primarily via stimulation of GnRH secretion [8, 15, 107], although direct effects at the level of the adenohypophysis have been demonstrated [141]. The ability of kisspeptin to stimulate LH release in prepubertal females [79, 135,

136] suggests that exogenous kisspeptin can override the gonadal deficiency of LH stimulation characteristic of immature states. In mature ewes, continuous infusion of kisspeptin sustains elevated circulating concentrations of LH for 24 h and stimulates ovulation during the anestrous season [69]. In this study, we determined the effects of intermittent injections of kisspeptin on the release of LH in prepubertal lambs. We also sought to test the hypothesis that repeated hourly administration of kisspeptin leads to synchronized hourly release of LH and stimulates follicular growth, ovulation and initiation of regular reproductive cycles.

Materials and Methods

Experimental procedures involving animals were performed at the Texas A&M University O.D. Buttler Animal Science Teaching and Research Complex, College Station, Texas. The Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System approved all procedures used in these studies.

Animals and experimental procedures

Twelve intact, spring-born ewe lambs, weaned at 8 wk of age, were used for this study. Ewe lambs were fed *ad libitum* a complete-mixed diet containing 16% CP and 2.8 MCal/kg formulated to promote weight gain of approximately 0.2 to 0.25 kg/d. Ewe lambs had free access to water and a mineral supplement. At 26 wk of age, lambs were brought into indoor pens and acclimated to experimental conditions (periodic loose restraint with halter) for two wk. At 28 wk of age, ewe lambs were assigned randomly

to one of two groups: 1) Control (n=6); or 2) Kisspeptin (n=6). On the day before onset of treatments, ewes were fitted with a jugular catheter. On the next day, ewes were injected i.v. with either saline (Control) or saline containing 20 µg of a decapeptide representing the carboxyl terminus of the ovine kisspeptin (Kisspeptin) [142] every 1 h for 24 h. Kisspeptin decapeptide was obtained by custom synthesis (American Peptide Company Inc., Sunnyvale, CA). The dose of kisspeptin used in this experiment was determined based on previous reports demonstrating the effectiveness of kisspeptin to stimulate release of LH in sheep [69].

Blood samples (5 ml) were collected from the jugular vein every 15 min starting 30 min before the first injection of saline or kisspeptin (Period 0), and continued for 6 h at 15-min intervals (Period I). Between 6 and 18 h from the onset of treatments (Period II), blood samples were collected just prior to each hourly injection. Collection of blood samples at 15-min intervals resumed 18 h after the start of treatments and continued for an additional 6 h (Period III). Following the 24 h administration of treatments, blood samples were collected every 3 h for 36 h (Period IV), daily for the next 15 d and then every 3 d until the ewe lambs were detected pubertal or reached 35 wk of age.

Intensive (every 15 min), hourly, and 3-hourly blood samples were placed in tubes containing 50 µl of a solution of heparin (3,000 U/ml) and 5% EDTA. Jugular catheters were flushed with physiological saline solution after every sampling and with heparinized saline every 1 h to prevent clotting of the catheter. Daily and 3-d blood samples were collected by jugular venipuncture. Blood samples were chilled on ice until centrifugation at 1500 x g for 20 min at 4^{o} C, and plasma or serum was collected and

stored at -20^{o} C until processed for determination of concentrations of LH and progesterone by RIA.

RIA

Concentrations of LH were determined in samples obtained during Periods 0 to IV by a double antibody RIA using rabbit anti-ovine LH (AFP192279; National Hormone and Pituitary Program, Torrance, CA) and ovine LH (AFP8614B; National Hormone and Pituitary Program) as the labeled tracer and reference preparation. The RIA procedures for LH were the same as stated earlier in Chapter III. Intraassay and interassay coefficients of variation averaged 11% and 13.5%, respectively. Concentrations of progesterone were determined in blood samples collected prior to the onset of treatments (Period 0), daily and every 3 d to detect luteal activity. Concentrations of progesterone were determined using a solid-phase RIA (Coat-A-Count; Diagnostic Products Corporation). Detection limit of the assay was 0.1 ng/ml. The intraassay and interassay coefficients of variation of serum pools producing displacement of tracer to approximately 50% (1.7 ng/ml) and 25% (7.5 ng/ml) of total binding averaged 7.5% and 24%, respectively.

Statistical analysis

Frequency and amplitude of LH pulses were determined using a pulse-detection algorithm (Pulsefit 1.2, [118]). Luteinizing hormone data were analyzed using the Proc Mixed procedures for repeated measures of SAS (SAS 9.1; SAS Institute, Cary, NC).

The main effects of treatment (saline or kisspeptin) on mean concentrations of LH and on the frequency and amplitude of LH pulses were compared using Period (I: 0-6 h, and III: 18-24 h) as repeated variable, and ewe(treatment) as the subject. The proportions of ewes in each treatment exhibiting a surge-like release of LH and elevated circulating progesterone 5 days after onset of treatments were analyzed by the Fisher's Exact Test procedure of SAS.

Results

One control ewe was observed to have elevated concentrations of progesterone on the first day of the experiment and data obtained from her were eliminated from further analysis. Mean (± SEM) body weight at the onset of treatments was 42.5 ± 1.7 kg and did not differ among treatment groups.

Mean concentrations of LH prior to the onset of treatments (Period 0) did not differ between treatments and averaged 1.9 ± 0.4 ng/ml. As expected, control ewes exhibited infrequent pulses of LH characteristic of peripubertal ewes (Fig. 4.1, Table 4.1). In the kisspeptin-treated ewes, a pulse of LH was observed within 15 min after every injection (Fig. 4.1), resulting in increased ($P < 0.02$) frequency of LH pulses compared to controls during the first 6 h after onset of treatments (Table 4.1). Similarly, mean concentrations of LH and mean amplitude of LH pulses were greater ($P < 0.005$) in kisspeptin-treated lambs compared to controls during Period 1 (Table 4.1).

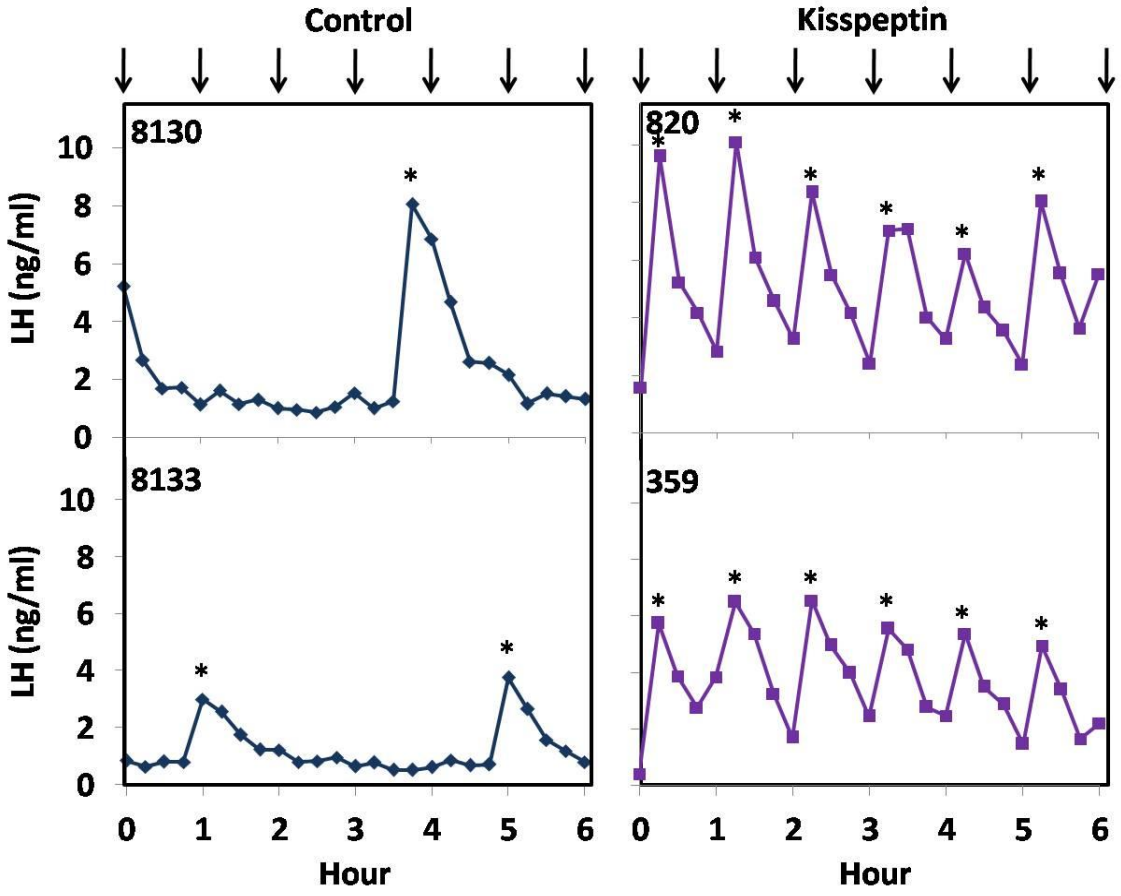

FIG. 4.1. Patterns of LH release in representative saline- (Control) and kisspeptin-treated ewe lambs during Period I (0 – 6 h) of the experiment. Control lambs exhibited lower frequency of LH pulses than kisspeptin-treated lambs. In kisspeptin-treated lambs, a pulse of LH was observed following every injection of kisspeptin. * Indicates detected pulses of LH. Arrows indicate the time of injections of saline (Control) or kisspeptin.

Table 4.1. Mean (±SEM) circulating concentrations of LH, and frequency and amplitude of LH pulses in saline- (Control) and kisspeptin-treated ewe lambs during Period I (0 – 6 h) of the experiment. Mean concentrations of LH, as well as frequency and amplitude of LH pulses were greater in kisspeptin-treated ewes.

	Control	Kisspeptin
Concentrations (ng/ml)	2.3 ± 0.1	4.5 ± 0.2*
Frequency of Pulses (pulses/6 h)	3.0 ± 0.7	6.0 ± 0.0**
Amplitude of Pulses (ng/ml)	3.1 ± 0.4	5.3 ± 0.4*

* $P < 0.005$
** $P < 0.02$

During Period III (18-24 h after onset of treatments), the pattern of release of LH in control ewe lambs remained similar to that of Period I, with only infrequent episodes of LH release observed. In the kisspeptin-treated group, four ewe lambs were observed to exhibit a surge release of LH beginning approximately 20 h after onset of kisspeptin injections (Table 4.2; Fig. 4.2). The time at onset of LH surges averaged (± SEM) 21 ± 2.3 h after onset of treatments. The mean maximum concentration of LH observed during the surge was 22 ± 13 ng/ml. The duration of the LH surge last approximately 12 h (Fig. 4.3). All ewes observed to exhibit a LH surge had elevated concentrations of progesterone (above 1 ng/ml) 5 days after the onset of treatments (Table 4.2), indicating that the surge of LH caused ovulation and/or follicle luteinization. Two of the kisspeptin-treated ewe lambs did not exhibit surge release of LH during the experiment,

although they continued to respond to the kisspeptin injections with a pulse-like release of LH within 15 min after injections (Fig. 4.2).

Table 4.2. Proportion of saline- (Control) and kisspeptin-treated ewe lambs exhibiting a surge release of LH and luteal activity within 5 days after onset of treatments. Four of six kisspeptin-treated lambs exhibited a surge rise in concentrations of LH approximately 20 h after the onset of kisspeptin injections and all were observed with circulating concentrations of progesterone above 1 ng/ml 5 d post treatment onset. None of the control ewes were observed to exhibit a LH surge or luteal activity during this period.

	Control	Kisspeptin
Preovulatory Surge	0/5	4/6 *
Luteal Activity	0/5	4/6 *

* $P < 0.05$

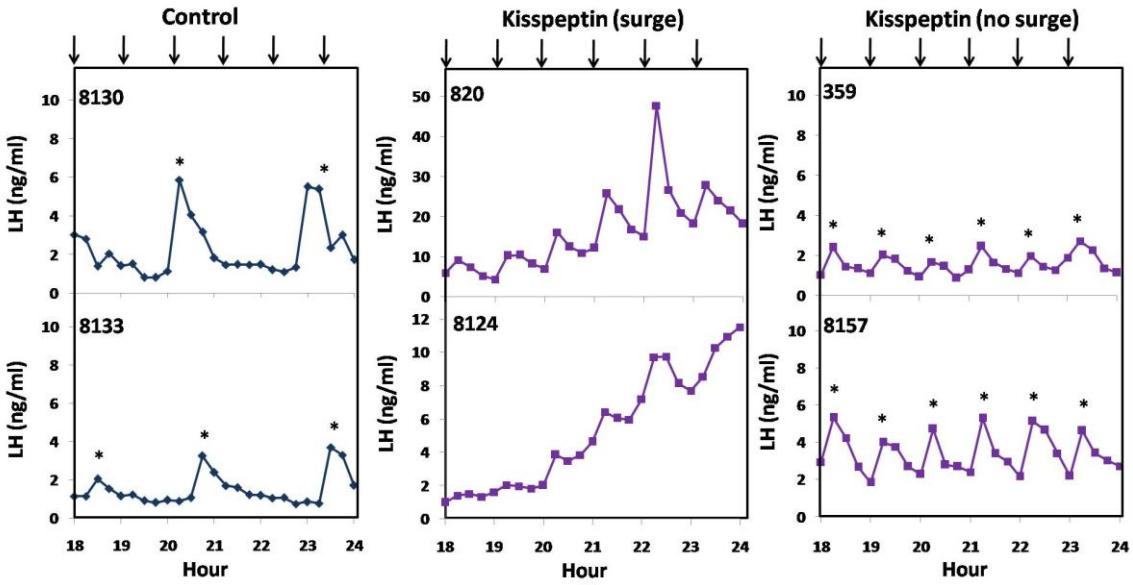

FIG. 4.2. Patterns of LH release in representative saline- (Control) and kisspeptin-treated ewe lambs during Period III (18 – 24 h) of the experiment. Patterns of LH release in control ewe lambs were similar to those during Period I. A surge release of LH was observed in three kisspeptin-treated ewes during Period III. The remaining three kisspeptin-treated lambs continued to respond to kisspeptin injections exhibiting pulses of LH within 15 min following treatments. * Indicates detected pulses of LH. Arrows indicate the time of injections of saline (Control) or kisspeptin.

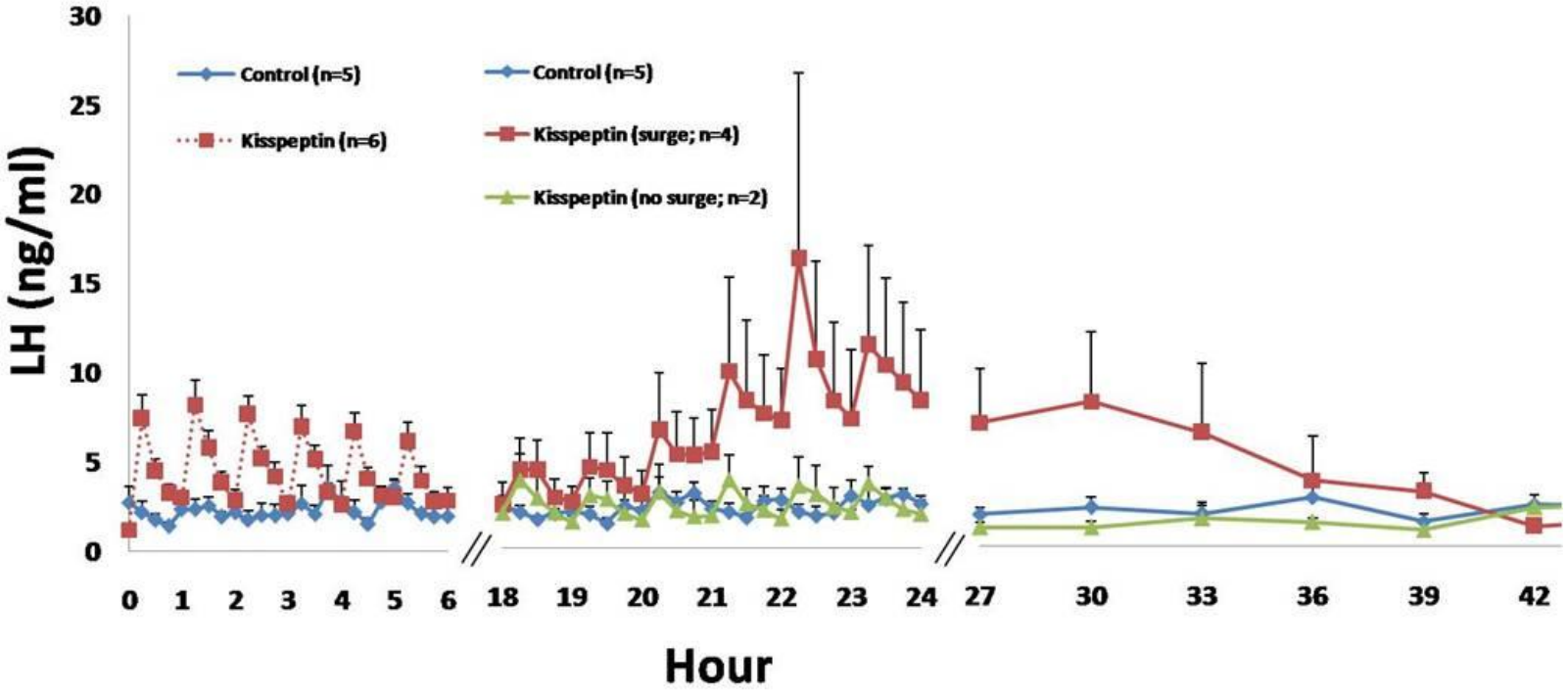

FIG. 4.3. Mean (± SEM) concentrations of LH in saline- (Control) and kisspeptin-treated ewe lambs. Hourly injections of 20 µg of kisspeptin in prepubertal ewe lambs stimulate the release of LH within 15 min following injections and leads to LH surge approximately 20 h after the onset of treatments.

In three of four kisspeptin-treated ewe lambs that exhibit luteal activity after treatments, concentrations of progesterone were elevated only briefly and decreased below 1 ng/ml 2 to 3 d after the initial rise. No control ewe lambs exhibited elevated concentrations of progesterone within 5 d following saline injections. The proportion of ewes determined pubertal in each group by 35 wk of age, defined as ewes exhibiting circulating concentrations of progesterone above 1 ng/ml for at least 5 days, did not differ between groups.

Discussion

The ability of kisspeptin to stimulate the release of LH in prepubertal female mammals has been demonstrated in rats [79], heifers [136] and gilts [135]. In the current study, we extended these findings by demonstrating that intermittent injections of kisspeptin elicit pulse-like release of LH in prepubertal ewe lambs. Moreover, hourly injections of kisspeptin administered for 24 h stimulated ovarian function in prepubertal lambs leading to stimulation of a preovulatory surge of LH, ovulation and luteal activity. The ability of kisspeptin to advance the onset of puberty has been suggested by observations that kisspeptin induces early vaginal canalization in rats, a physiological event that precedes the first estrous cycle in female rats [79]. Although in our studies kisspeptin treatment stimulated ovulation in prepubertal ewes, duration of luteal activity was brief (2 to 3 days), and the age at establishment of regular estrous cycles was not advanced as hypothesized.

Kisspeptin is a potent stimulator of LH release in various mammalian and non-mammalian species (for review, see [100, 143, 144]). Although our studies did not distinguish the site of kisspeptin actions, the effects of kisspeptin on stimulating the release of LH in prepubertal ewe lambs are likely to be mediated by direct actions upon GnRH neurons. Kisspeptin receptor (GPR54) is present in GnRH neurons [8, 13], and kisspeptin stimulates the release of GnRH [8]. Moreover, kisspeptin-stimulation of LH release is blocked by GnRH antagonist [68, 79, 107] and passive immunization with GnRH antiserum [142]. However, direct effects of kisspeptin in the hypophysis to stimulate LH release cannot be disregarded. Kisspeptin receptor is also expressed in various tissues, including the adenohypophysis [34, 35], and in vitro studies demonstrate that kisspeptin induces LH release in primary culture of adenohypophyseal cells [34, 36]. Nevertheless, the physiological relevance for the direct effects of kisspeptin in the adenohypophysis remains controversial, because kisspeptin failed to stimulate release of LH in hypothalamo-hypophyseal disconnected ewes [34].

In the current study, repetitive hourly injections of kisspeptin in prepubertal ewe lambs elicited episodes of LH release similarly to intermittent injections of kisspeptin in agonadal, male monkeys [68]. In control-treated ewe lambs, spontaneous pulses of LH were observed, indicating that endogenous pulses of LH could be detected in ewe lambs at this age. Because all episodes of LH release observed in kisspeptin-treated lambs occurred immediately after kisspeptin treatments and no additional LH pulses between kisspeptin injections were detected, we speculate that kisspeptin may be involved in the neuronal pathways leading to synchronized episodic release of GnRH. Although there is

evidence to indicate that GnRH neurons have an intrinsic ability to generate pulses [145, 146], pulses of GnRH release may be entrained by actions of various neurochemicals, including neurotransmitters and glial-derived factors [147, 148]. Kisspeptin has been shown to act directly on GnRH neurons to increase neuronal activity [71]. Therefore, kisspeptin transmission may play a role in synchronizing GnRH neuronal activity leading to episodic neurohormone release.

The mode of kisspeptin treatment used in the current studies differs from other studies using constant infusion [8, 14, 69]. In monkeys, after an initial increase, concentrations of LH decreased with continuous infusion of kisspeptin and were no different than controls within 24 h [67]. Although a single bolus of kisspeptin failed to release LH during continued administration of kisspeptin, NMDA, a known GnRH secretagogue, and GnRH elicited LH release [67]. This observation indicates that kisspeptin receptor is desensitized by continuous kisspeptin treatment. The mechanisms of kisspeptin receptor desensitization are not clear, but seem to involve GPCR kinase 2 [149]. In sheep, concentrations of LH also decrease during continuous infusion of kisspeptin [8]; however, low doses of kisspeptin infused constantly increase concentrations of estradiol in circulation and stimulate ovulation in anestrous ewes [69, 150]. Nevertheless, endogenous kisspeptin release in the median eminence seems to be episodic [131] and this mode of action may be relevant for the control of GnRH neuronal activity.

It has been proposed that proper activation of the kisspeptin system is essential for normal pubertal onset [13-15]. Increases in kisspeptin gene expression [14, 15] and

peptide [151] have been associated with pubertal development. In ewe lambs, initiation of frequent pulsatile LH release is associated with increases in expression of *Kiss1* in the arcuate nucleus (see Chapter III). Therefore, kisspeptin stimulation of GnRH neuronal function may be critical for pubertal transition toward patterns of pulsatile LH release that support final stages of follicle development (approximately 1 pulse/h in the sheep) and a prerequisite for establishment of normal regular estrous cycles.

Luteinizing hormone stimulation is crucial for increased follicular estradiol synthesis and sustained elevated concentrations of estradiol that lead to the preovulatory surge of LH. The ability of estradiol to induce LH surge develops early during the juvenile period [22, 152]. Thus, estradiol positive feedback is not the limiting factor in the initiation of regular ovulatory cycles. In 18-wk-old ewes, hourly injections of LH for 48 h stimulated elevations in circulating estradiol and surges of LH occurred between 23 and 55 h after initiation of LH injections in four of seven lambs [21]. Ovulation was followed by increases in circulating concentrations of progesterone that remained elevated for a full normal-length luteal phase in only one lamb [30]. Similar results were observed in the current study using hourly injections of kisspeptin, in which approximately 70% of kisspeptin-treated ewe lambs ovulated. Therefore, although follicular development, increased steroidogenesis and ovulation were achieved, establishment of full-length luteal phase and regular estrous cycles may require maturation of neuroendocrine functions upstream to GnRH neurons.

There is compelling evidence for a role of kisspeptin in mediating estradiol positive feedback on GnRH release. In rodents, the population of kisspeptin neurons in

the AVPV seems to be particularly relevant. Estradiol stimulates *Kiss1* expression in the AVPV [10], kisspeptin neurons in the AVPV express the immediate-early gene *cFos* during LH surge [127] and immunoneutralization of kisspeptin in the POA blocks the preovulatory surge of GnRH [102]. In sheep, the involvement of POA kisspeptin neurons in the estradiol positive feedback is less clear. Recent observations suggest that kisspeptin neurons in the caudal arcuate nucleus are involved in the estradiol positive feedback [125]. Nevertheless, kisspeptin neurons in the POA of sheep may also be involved in the preovulatory surge of GnRH/LH because *Kiss1* expression increases in the POA of ewes just before ovulation. Moreover, increase in cFos immunoreactivity was observed in POA kisspeptin neurons at the time of the surge [Caraty et al., personal communications]. During the preovulatory LH surge, approximately 40% of GnRH neurons contain immunoreactive Fos [123, 153], suggesting that only a subpopulation of GnRH neurons are activated in response to the estradiol-induced LH surge. The preovulatory surge of GnRH/LH is characterized by a massive release of LH and lack of distinctly detectable pulses [154]. In the current study, two of the ewe lambs had the onset of the LH surge during the second period of intensive blood sampling at the end of kisspeptin treatment. Interestingly, despite escalating concentrations of LH in circulation, clear elevations in LH were observed in association with kisspeptin injections. This observation may reflect differential actions of kisspeptin in distinct populations of GnRH neurons. In sheep, GnRH neurons dispersed throughout the preoptic area and hypothalamus become activated during the LH surge [123], whereas

GnRH neurons residing mainly in the mediobasal hypothalamus are activated during the pulsatile release of LH [130].

Formation of the corpus luteum after ovulation and initial secretion of progesterone does not require hypophyseal hormones [155]. However, adequate LH stimulation is required for maintenance of normal luteal function [156]. In the current experiments, ewes that ovulated following kisspeptin treatment had only a brief period of elevated progesterone. This short period of luteal activity may reflect insufficient release of LH after termination of kisspeptin treatment. Although short luteal phase is commonly observed in ewes undergoing pubertal transition, it is often followed immediately by another ovulation and a normal-length estrous cycle [18]. The lack of immediate establishment of regular estrous cycles in ewe lambs ovulating following kisspeptin treatment suggests that the reproductive neuroendocrine axis was not yet fully mature.

In summary, injection of kisspeptin stimulates the release of LH in prepubertal ewe lambs. Intermittent treatments with kisspeptin that create hourly pulses of LH stimulate folliculogenesis and steroidogenesis, and lead to development of the preovulatory LH surge and ovulation. However, luteal activity following induced ovulation is limited and regular estrous cycles are not established immediately in absence of kisspeptin treatments. Therefore, kisspeptin seems to play a major role in the maturation of the hypothalamic-gonadotropic-gonadal axis by stimulating GnRH neuron activity and pulsatile release of LH during the onset of puberty in ewe lambs.

CHAPTER V

SUMMARY AND CONCLUSIONS

The studies reported herein support a role for kisspeptin in the regulation of reproductive maturation in the female sheep. The number of cells expressing *Kiss1* increased in both the POA and ARC during juvenile maturation in the ewe lamb. Furthermore, the increase in number of *Kiss1*-expressing cells in the ARC is strongly correlated with the increase in the frequency of LH pulses that occur during maturation of the reproductive neuroendocrine axis. This suggests that the POA and ARC populations of kisspeptin neurons may be relevant for stimulating the pattern of LH release that is required for the onset of puberty in mammals. This action is likely through the stimulation of GnRH neurons. Interestingly, no increase in *Kiss1* expression per cell was observed. The mechanisms that drive the *Kiss1* gene to be turned on during pubertal maturation remain to be elucidated.

The observations that injections of kisspeptin activated the hypothalamic-pituitary-gonadal axis in 28-wk-old, prepubertal ewe lambs support further a role for kisspeptin in the onset of puberty. Intermittent injections of kisspeptin elevated the frequency of LH pulses and the majority of ewe lambs treated with kisspeptin ovulated within 30 h following onset of injections. However, activation of reproductive axis in prepubertal lambs was temporary and the onset of estrous cycles did not occur immediately following kisspeptin-induced ovulation. Consequently, injections of kisspeptin did not affect age at puberty in our studies.

In conclusion, our studies suggest that lack of adequate synthesis and release of kisspeptin is a primary limiting factor for the onset of mature patterns of LH release in prepubertal lambs. The mechanisms that support enhanced *Kiss1* gene expression during pubertal transition remain unknown. However, pharmaceutical approaches using kisspeptin and kisspeptin analogues can be used to stimulate and/or regulate the release of LH from the pituitary gland. Approaches include the use of kisspeptin to induce ovulation in peripubertal and anestrous, mature ewes.

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APPENDIX A

LUTEINIZING HORMONE GRAPHS FOR EXPERIMENTS 1 AND 2

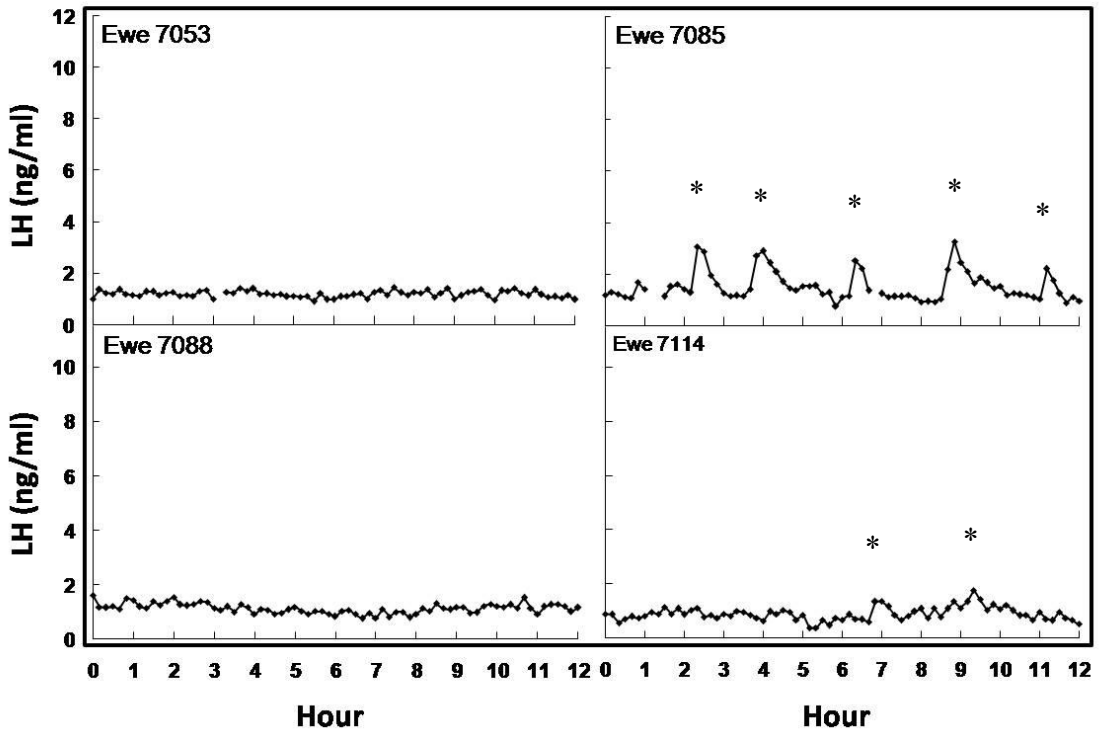

Fig A-1. Patterns of LH release for 25-wk-old ewes not shown in Chapter III.

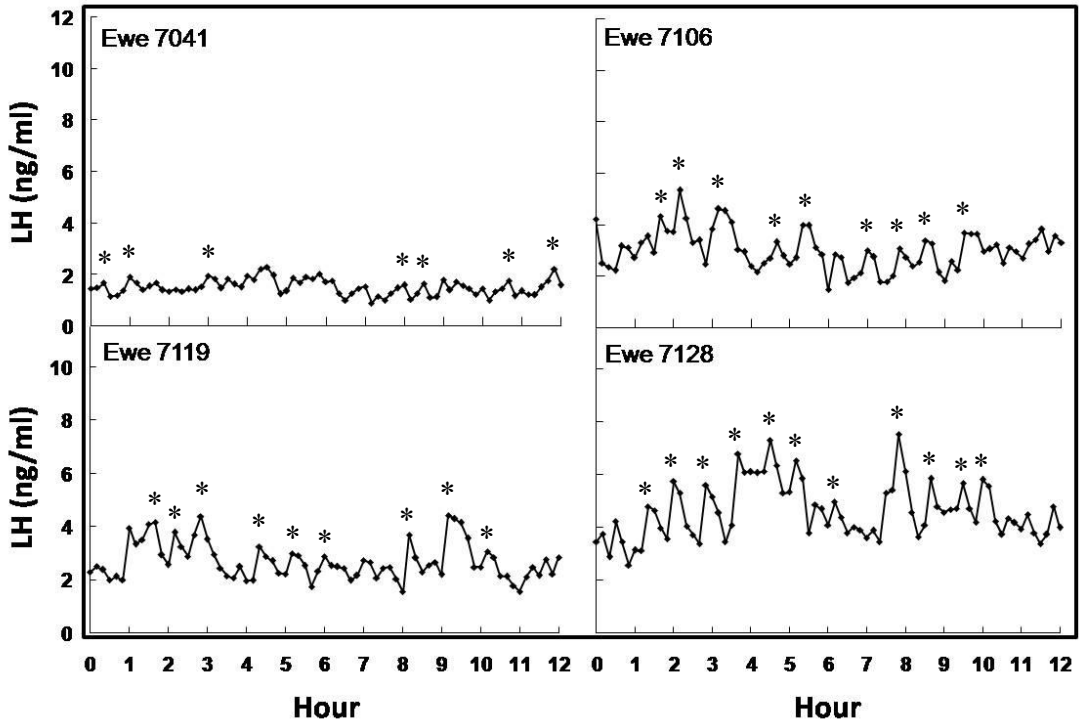

Fig A-2. Patterns of LH release for 35-wk-old ewes not shown in Chapter III.

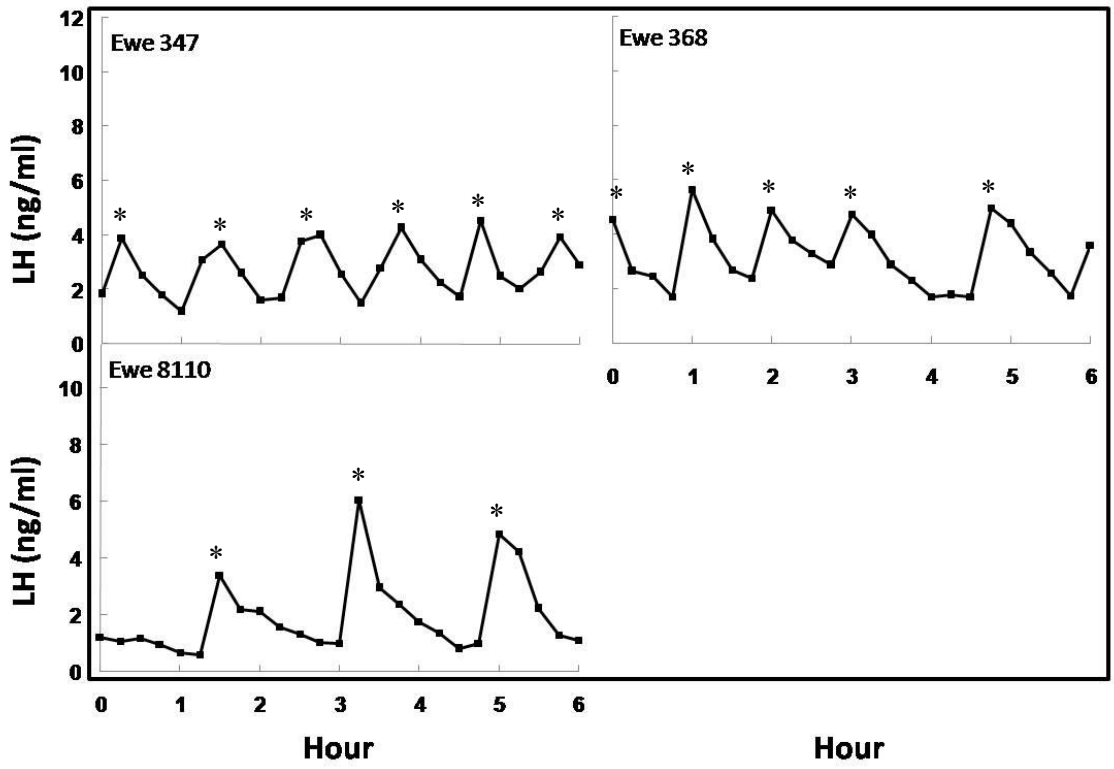

Fig A-3. Patterns of LH release in control ewes not shown in Chapter IV during Period I.

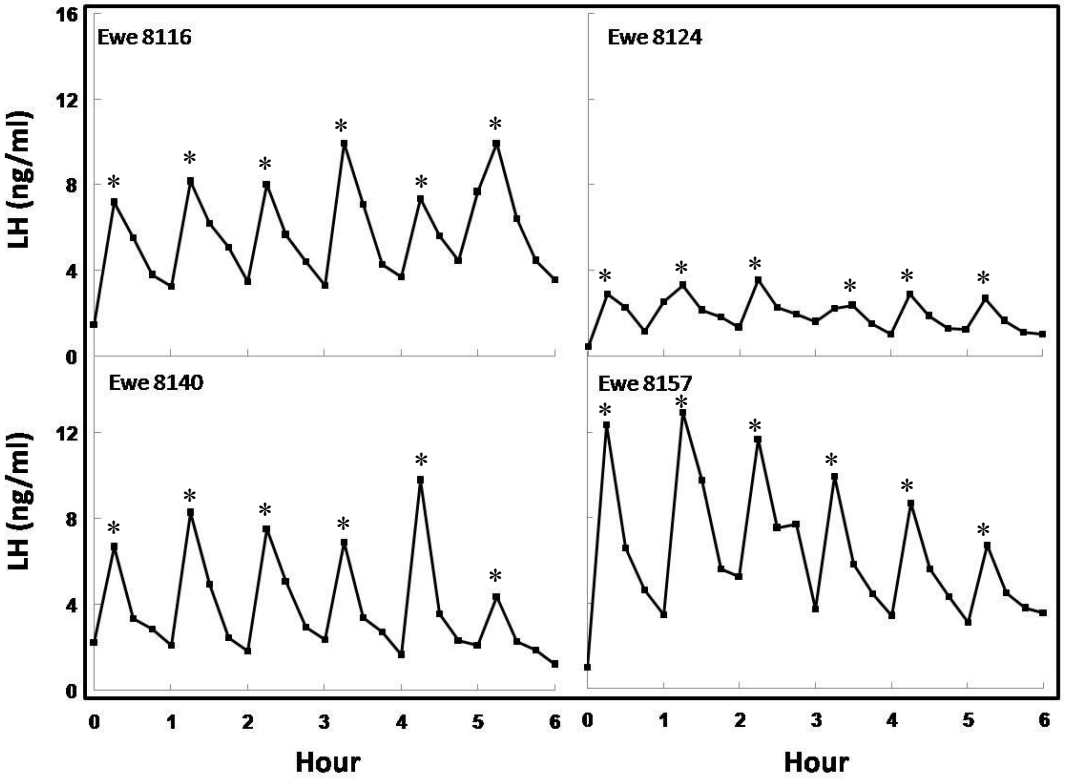

Fig A-4. Patterns of LH release in kisspeptin-treated ewes not shown in Chapter IV during Period I.

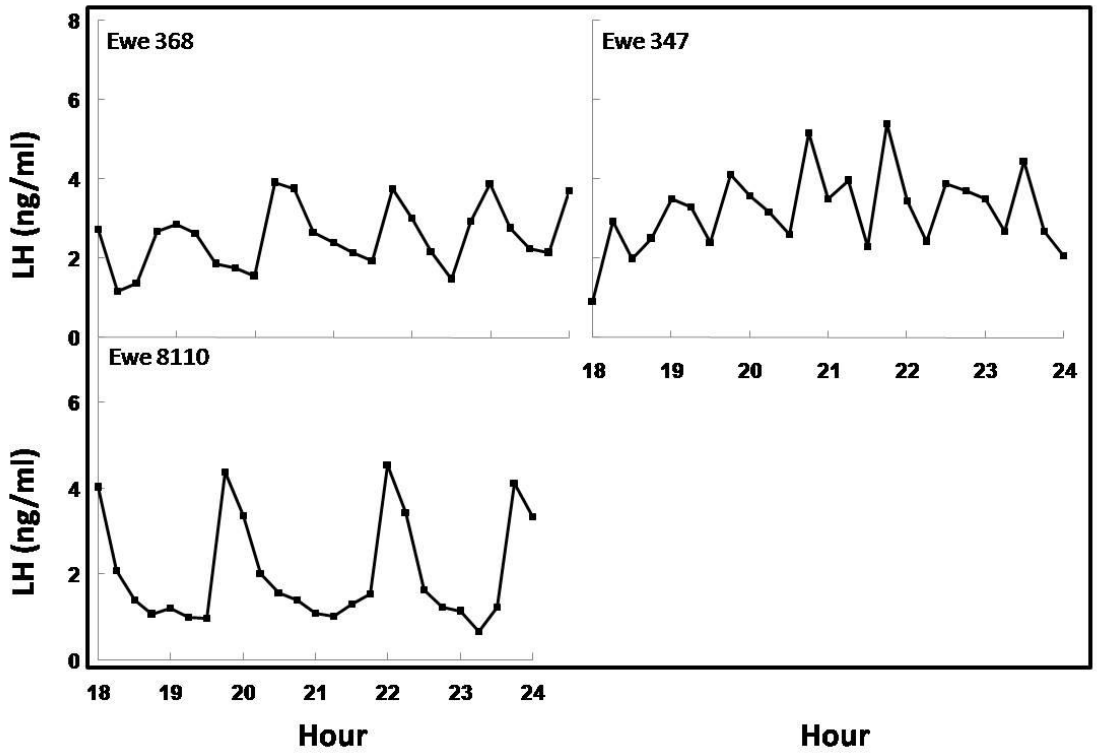

Fig A-5. Patterns of LH release in control ewes not shown in Chapter IV during Period III.

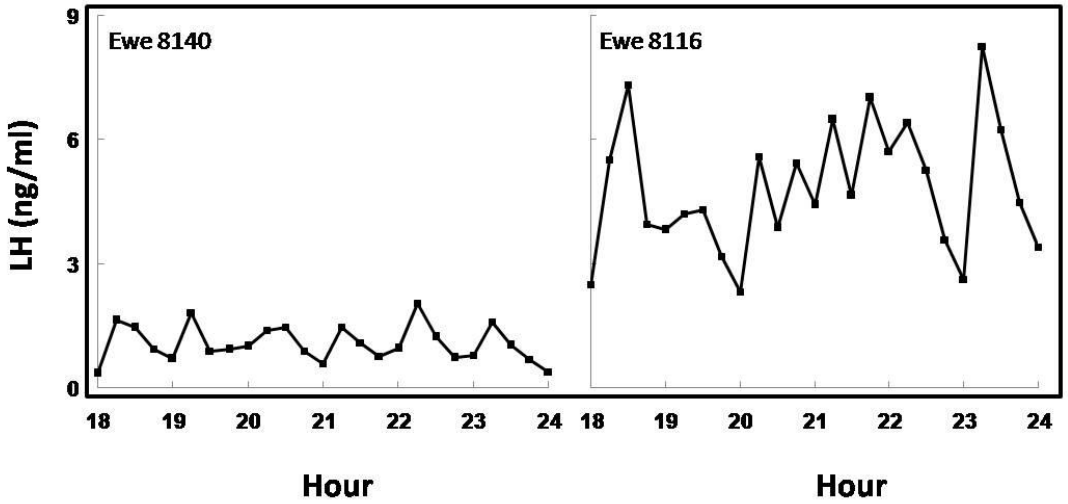

Fig A-6. Patterns of LH release in kisspeptin-treated ewes not shown in Chapter IV during Period III.

APPENDIX B

LABORATORY PROCEDURES

Procedure B-1. *Kiss1* In Situ Hybridization

Probe Synthesis: MAXIscript® Procedure (Ambion/Applied Biosystems)
Sense- Control probe; Antisense- Detection probe

Hybridization: All dishware and solutions must be RNase free.

a) **Day 1: Hybridization**
1. Air dry slides for 2 min using a blow dryer
2. Fix sections in 4% PAF diluted in 0.1M PB for 15 min with stirring at RT
3. Wash in 0.1M PB for 5 min twice at RT
4. Incubate slides in Proteinase K for 30 min at 37 ^{o}C
5. Wash slides in 0.1M PB at RT
6. Return slides to 4% PAF for 5 min with stirring at RT
7. Wash slides in 0.1M PB at RT
8. Dip slides in DEPC water twice
9. Dip slides in TEA once
10. Dip slides in TEA containing acetic anhydride for 10 min with stirring at RT
11. Wash slides in 2X SSC
12. Dehydrate sections through 70%, 95% , and 100% EtOH for 3 min each
13. Delipidate sections in chloroform for 5 min
14. Wash in 100% and 95% EtOH for 3 min each; air dry

15. Denature radiolabeled cRNA probe ($1x10^6$ cpm/150 µl) in hybridization buffer containing 100 mM DTT at 70 ^{o}C for 10 min
16. Let solution cool on ice for 5 min
17. Add 150 µl hybridization solution to the middle of each slide
18. Gently place parafilm coverslip on each slide – avoid bubbles
19. Hybridize overnight at 55 ^{o}C in humidified chamber containing Whatman 3 MM paper wetted with 50% formamide + 5X SSC

b) **Day 2: Washing**
1. Remove parafilm coverslips from slides and place slides on rack in 5X SSC
2. Wash slides in the following:
   i. 5X SSC containing 10mM βME for 30 min at 55 ^{o}C
   ii. 1X TEN buffer for 10 min, 3 times, at 37 ^{o}C
   iii. RNase in TEN buffer for 30 min at 37 ^{o}C
   iv. TEN buffer for 30 min at 37 ^{o}C
   v. 2X SSC containing 10mM βME for 30 min at 55 ^{o}C, repeat for 15 min

vi. 0.1X SSC containing 10mM βME for 15 min at 55 ^{o}C
vii. 0.1X SSC for 15 min at RT

3. Dehydrate sections through the following:
   i. 70% EtOH + 0.3M NH_4Ac for 5 min, twice
   ii. 90% EtOH + 0.3M NH_4Ac for 3 min
   iii. 100% EtOH for 2 min, twice
4. Air dry slides 1-3h
5. Expose slides to film overnight to estimate autoradiography time

c) **Day 3: Autoradiography**

1. Warm photographic NBT2 emulsion in a light tight container to 44^{o} C in a water bath
2. Warm equal volume of ddH_20 to 44^{o} C in a water bath
3. Place a dipping chamber into a water bath set at 44^{o} C

In the dark (with safe-light, if preferred)

4. Mix photographic NBT2 emulsion with ddH_20 in a 1:1 dilution
5. Pour emulsion into the dipping chamber and let it equilibrate to 42 - 44^{o} C
6. Dip each slide once and withdrawal it slowly from the emulsion
7. Keep slides in vertical position and wipe excess emulsion from the back of slide
8. Let slides to stand up in a rack and air dry overnight (leave safe-light off during this time or place slide rack in a light-tight box)
9. Place slides in a plastic slide box containing desiccant
10. Wrap slide box in double foil to avoid light exposure and place at 4^{o} C for appropriate amount of time

d) **Day 4: Developing Slides**

1. Allow slides to warm to room temperature
2. Chill Kodak D-19 Developer to 15^{o} C and dilute 1:1

In the dark (with safe-light, if preferred, at least 1.5 m from slides)

3. Place slides into chilled D19 Developer (diluted 1:1 with ddH_2O) for 4 min – dip approx 10 times/min
   * Do not use developer solution for more than one rack
4. Dip in ddH_2O for 30 sec to stop development
5. Place slides into Kodak Fixer (with hardener, diluted 1:7 with ddH_2O) for 5 min
6. Dip in ddH_2O for 5 min (can turn lights on)

Counterstaining & Coverslipping

1. Place slides in 1X Acetate buffer for 30 sec or longer

2. Place slides in Cresyl Violet (filtered before use) for 15 min
3. Dip slides once in acidified 70% ETOH (do not use for more than 2 racks)
4. Dip slides 4 times in 70% ETOH
5. Dip slides for 45 seconds in 90% ETOH
6. Place slides in 100% ETOH for 3 min
7. Place slides in Citrosolv 3 times for 5 min each
8. Coverslip using DPX and 24 x 60 mm glass coverslips

Procedure B-2. Luteinizing Hormone RIA

1. Iodination: Iodination grade oLH (AFP-8614B; NHPP)
   Reaction: 10 µg of hormone, 0.5 mCi of ^{125}I, 80 µg Chloramine T, 2 min

2. Antibody: Anti-ovine LH (rabbit anti-oLH – AFP-192279; obtained from Dr. A.F. Parlow
   Dilution: 1:1,000,000

3. Standards: Iodination grade oLH (AFP-8614B)
   Range: 0.0625 – 50 ng/ml

4. References preparation: oLH added to ewe serum

5. RIA procedure
   a) Complete assay sheets and label polypropelene tubes
      5 TC, 4 NSB, 3 "0", standards in triplicate, 2 x references in duplicate, and unknown samples in duplicate

   b) Day 1: Pipette the following into each tube-
      NSB: 500 µl PBS-1% EW
      0 std: 500 µl PBS-1% EW
      Stds: 200 µl standard + 300 µl PBS-1% EW
      Refs: 200 µl reference + 300 µl PBS-1% EW
      Unknowns: 200 µl sample + 300 µl PBS-1% EW

      Pipette 200 µl PBS-EDTA + 1:400 NRS without 1st Ab into NSB tubes
      Pipette 200 µl anti-oLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except TC and NSB
      Vortex briefly and incubate at room temperature for 1-2 h

      Pipette 100 µl ^{125}I-oLH (20,000 cpm/tube diluted in PBS-1% EW) into all tubes
      Vortex briefly and incubate at 4^{o} C for 20-24 h

   c) Day 2: Pipette 200 µl of goat-anti-rabbit gamma globulin (GAR; 2nd Ab diluted in PBS-EDTA into all tubes except TC
      Vortex and incubate at 4^{o} C for 48-72 h

   d) Day 4: On per spin basis, add 3 ml cold 0.01M PBS into all tubes except TC
      Centrifuge tubes at 3600 rpm at 4^{o} C for 1 h
      Decant supernatant
      Count radioactivity associated with the pellet in gamma counter

Procedure B-3. Progesterone RIA

Single Antibody RIA Kit, Diagnostic Products Corporation, Los Angeles, CA
References:
Jones et al., 1991. J. Anim. Sci. 69:1607
Simpson et al., 1992. J. Anim. Sci. 70:1478.

1. Iodinated Product: Iodination grade hP4.

2. Antibody: Anti-human P4 coated tubes.

3. Standards: Human serum with added P4. Range: 0.1 – 20.0 ng/ml.

4. Reference: Human standard preparation added to bovine serum.

5. RIA Procedure:

A. Conduct assay

1) Pipette in non-coated polypropylene tubes

NSB – 100 µl of 0 std

2) Pipette in antibody coated tubes

0 Std – 100 µl
Std – 100 µl
Ref – 100 µl
Unknowns – 100 µl

3) Pipette 1 ml of 125I-P4 provided in the kit into all tubes including three Total Count non-coated polypropylene tubes.

4) Vortex tubes briefly and incubate at room temperature for 3 h.

5) Pour off supernatant.

6) Count radioactivity using gamma counter.

APPENDIX C

STATISTICAL ANALYSIS DATA AND COMMANDS

Table C-1. Statistical analysis of means for variables in Experiment 1.

trt	N Obs	Variable	Mean	Std Dev	Std Error
25	6	bw	38.1666667	7.2502874	2.9599174
		lh	1.0333333	0.3076795	0.1256096
		lhfreq	1.6666667	2.0655911	0.8432740
		lhamp	0.5666667	0.6947422	0.2836273
		poatot	124.6666667	53.4889397	21.8367682
		sixarctot	117.3333333	29.6085573	12.0876429
		poaden	4470.83	1320.83	539.2278069
		arcden	4817.67	988.9308705	403.7293373
30	6	bw	44.1666667	7.0261417	2.8684103
		lh	2.3000000	1.4642404	0.5977736
		lhfreq	4.5000000	4.4609416	1.8211718
		lhamp	1.8666667	1.5590595	0.6364834
		poatot	192.2000000	45.1962388	20.2123724
		sixarctot	176.3333333	131.2397298	53.5783953
		poaden	4878.80	1315.43	588.2802393
		arcden	5383.83	628.3194782	256.5103528
35	6	bw	55.1666667	5.8109093	2.3722938
		lh	3.4666667	1.3109793	0.5352050
		lhfreq	10.5000000	3.1464265	1.2845233
		lhamp	1.8500000	0.8167007	0.3334167
		poatot	193.5000000	41.1861627	16.8141805
		sixarctot	228.5000000	155.9394113	63.6619981
		poaden	4779.17	801.2219210	327.0974795
		arcden	5527.33	1351.13	551.5971155

Table C-2. Statistical analysis of mean bodyweight in Experiment 1.

The GLM Procedure

t Tests (LSD) for bw

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	45.23333
Critical Value of t	2.13145
Least Significant Difference	8.2764

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	55.167	6	35
B	44.167	6	30
B			
B	38.167	6	25

Least Squares Means

trt	bw LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	38.1666667	2.7457037	<.0001	1
30	44.1666667	2.7457037	<.0001	2
35	55.1666667	2.7457037	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: bw

i/j	1	2	3
1		0.1431	0.0005
2	0.1431		0.0126
3	0.0005	0.0126	

Table C-3. Statistical analysis of mean circulating concentrations of LH in Experiment 1.

The GLM Procedure

Dependent Variable: lh

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	17.77333333	8.88666667	6.74	0.0082
Error	15	19.78666667	1.31911111		
Corrected Total	17	37.56000000			

R-Square	Coeff Var	Root MSE	lh Mean
0.473198	50.67025	1.148526	2.266667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	17.77333333	8.88666667	6.74	0.0082

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	17.77333333	8.88666667	6.74	0.0082

t Tests (LSD) for lh

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1.319111
Critical Value of t	2.13145
Least Significant Difference	1.4134

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	3.4667	6	35
A			
B A	2.3000	6	30
B			
B	1.0333	6	25

Table C-3 (cont.).

Least Squares Means

trt	lh LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	1.03333333	0.46888362	0.0436	1
30	2.30000000	0.46888362	0.0002	2
35	3.46666667	0.46888362	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lh

i/j	1	2	3
1		0.0754	0.0023
2	0.0754		0.0989
3	0.0023	0.0989	

Table C-4. Statistical analysis of frequency of LH pulses in Experiment 1.

The GLM Procedure

Dependent Variable: lhfreq

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	244.1111111	122.0555556	10.75	0.0013
Error	15	170.3333333	11.3555556		
Corrected Total	17	414.4444444			

R-Square	Coeff Var	Root MSE	lhfreq Mean
0.589008	60.65641	3.369801	5.555556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	244.1111111	122.0555556	10.75	0.0013

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	244.1111111	122.0555556	10.75	0.0013

t Tests (LSD) for lhfreq

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	11.35556
Critical Value of t	2.13145
Least Significant Difference	4.1469

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	10.500	6	35
B	4.500	6	30
B			
B	1.667	6	25

Table C-4 (cont.).

trt	lhfreq LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	1.6666667	1.3757153	0.2444	1
30	4.5000000	1.3757153	0.0052	2
35	10.5000000	1.3757153	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lhfreq

i/j	1	2	3
1		0.1659	0.0004
2	0.1659		0.0076
3	0.0004	0.0076	

Table C-5. Statistical analysis of amplitude of LH pulses in Experiment 1.

The GLM Procedure

Dependent Variable: lhamp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	6.67444444	3.33722222	2.80	0.0929
Error	15	17.90166667	1.19344444		
Corrected Total	17	24.57611111			

R-Square	Coeff Var	Root MSE	lhamp Mean
0.271583	76.51393	1.092449	1.427778

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	6.67444444	3.33722222	2.80	0.0929

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	6.67444444	3.33722222	2.80	0.0929

t Tests (LSD) for lhamp

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1.193444
Critical Value of t	2.13145
Least Significant Difference	1.3444

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	1.8667	6	30
A			
A	1.8500	6	35
A			
A	0.5667	6	25

Table C-5 (cont.).

trt	lhamp LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	0.56666667	0.44599037	0.2232	1
30	1.86666667	0.44599037	0.0008	2
35	1.85000000	0.44599037	0.0009	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lhamp

i/j	1	2	3
1		0.0571	0.0600
2	0.0571		0.9793
3	0.0600	0.9793	

Table C-6. Statistical analysis of POA cell counts in Experiment 1.

The GLM Procedure

Dependent Variable: poatot

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	18084.83725	9042.41863	4.09	0.0399
Error	14	30957.63333	2211.25952		
Corrected Total	16	49042.47059			

R-Square	Coeff Var	Root MSE	poatot Mean
0.368759	27.85396	47.02403	168.8235

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	18084.83725	9042.41863	4.09	0.0399

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	18084.83725	9042.41863	4.09	0.0399

t Tests (LSD) for poatot

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	2211.26
Critical Value of t	2.14479

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
35 - 30	1.30	-59.77	62.37	
35 - 25	68.83	10.60	127.06	***
30 - 35	-1.30	-62.37	59.77	
30 - 25	67.53	6.46	128.60	***
25 - 35	-68.83	-127.06	-10.60	***
25 - 30	-67.53	-128.60	-6.46	***

Table C-6 (cont.).

Least Squares Means

trt	poatot LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	124.666667	19.197480	<.0001	1
30	192.200000	21.029786	<.0001	2
35	193.500000	19.197480	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: poatot

i/j	1	2	3
1		0.0326	0.0238
2	0.0326		0.9642
3	0.0238	0.9642	

Table C-7. Statistical analysis of POA cell density for Experiment 1.

The GLM Procedure

Dependent Variable: poaden

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	512553.42	256276.71	0.19	0.8288
Error	14	18854254.47	1346732.46		
Corrected Total	16	19366807.88			

R-Square	Coeff Var	Root MSE	poaden Mean
0.026466	24.69309	1160.488	4699.647

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	512553.4157	256276.7078	0.19	0.8288

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	512553.4157	256276.7078	0.19	0.8288

t Tests (LSD) for poaden

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	1346732
Critical Value of t	2.14479

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits	
30 - 35	99.6	-1407.5	1606.8
30 - 25	408.0	-1099.2	1915.1
35 - 30	-99.6	-1606.8	1407.5
35 - 25	308.3	-1128.7	1745.4
25 - 30	-408.0	-1915.1	1099.2
25 - 35	-308.3	-1745.4	1128.7

Table C-7 (cont.).

trt	poaden LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	4470.83333	473.76725	<.0001	1
30	4878.80000	518.98602	<.0001	2
35	4779.16667	473.76725	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: poaden

i/j	1	2	3
1		0.5708	0.6524
2	0.5708		0.8893
3	0.6524	0.8893	

Table C-8. Statistical analysis of ARC cell counts for Experiment 1.

The GLM Procedure

Dependent Variable: sixarctot

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	37120.7778	18560.3889	1.31	0.2983
Error	15	212088.1667	14139.2111		
Corrected Total	17	249208.9444			

R-Square	Coeff Var	Root MSE	sixarctot Mean
0.148954	68.31636	118.9084	174.0556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	37120.77778	18560.38889	1.31	0.2983

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	37120.77778	18560.38889	1.31	0.2983

t Tests (LSD) for sixarctot

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	14139.21
Critical Value of t	2.13145
Least Significant Difference	146.33

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	228.50	6	35
A			
A	176.33	6	30
A			
A	117.33	6	25

Table C-8 (cont.).

Least Squares Means

trt	sixarctot LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	117.333333	48.544157	0.0288	1
30	176.333333	48.544157	0.0025	2
35	228.500000	48.544157	0.0003	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sixarctot

i/j	1	2	3
1		0.4036	0.1262
2	0.4036		0.4591
3	0.1262	0.4591	

Table C-9. Statistical analysis of ARC cell density for Experiment 1.

The GLM Procedure

Dependent Variable: arcden

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1689527.44	844763.72	0.79	0.4708
Error	15	15991629.50	1066108.63		
Corrected Total	17	17681156.94			

R-Square	Coeff Var	Root MSE	arcden Mean
0.095555	19.69362	1032.525	5242.944

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	1689527.444	844763.722	0.79	0.4708

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	1689527.444	844763.722	0.79	0.4708

t Tests (LSD) for arcden

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1066109
Critical Value of t	2.13145
Least Significant Difference	1270.6

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	5527.3	6	35
A			
A	5383.8	6	30
A			
A	4817.7	6	25

Table C-9 (cont.).

trt	arcden LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
25	4817.66667	421.52672	<.0001	1
30	5383.83333	421.52672	<.0001	2
35	5527.33333	421.52672	<.0001	3

Least Squares Means for effect trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: arcden

i/j	1	2	3
1		0.3573	0.2524
2	0.3573		0.8130
3	0.2524	0.8130	

Table C-10. Correlation analysis between variables in Experiment 1.

The CORR Procedure

8 Variables: bw lh lhamp lhfreq poatot sixarctot poaden arcden

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
bw	18	45.83333	9.61157	825.00000	29.00000	61.00000
lh	18	2.26667	1.48641	40.80000	0.60000	5.00000
lhamp	18	1.42778	1.20235	25.70000	0	3.70000
lhfreq	18	5.55556	4.93752	100.00000	0	14.00000
poatot	17	168.82353	55.36384	2870	51.00000	268.00000
sixarctot	18	174.05556	121.07580	3133	20.00000	449.00000
poaden	17	4700	1100	79894	2209	6571
arcden	18	5243	1020	94373	2865	6473

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	bw	lh	lhamp	lhfreq	poatot	sixarctot	poaden	arcden
bw	1.00000	0.58425	0.56949	0.73709	0.07923	0.47141	0.09810	0.51483
		0.0109	0.0136	0.0005	0.7625	0.0483	0.7080	0.0288
	18	18	18	18	17	18	17	18
lh	0.58425	1.00000	0.74835	0.89154	0.35082	0.63218	0.10008	0.56995
	0.0109		0.0004	<.0001	0.1674	0.0049	0.7023	0.0135
	18	18	18	18	17	18	17	18
lhamp	0.56949	0.74835	1.00000	0.78894	0.30058	0.52706	0.15677	0.62101
	0.0136	0.0004		<.0001	0.2411	0.0246	0.5479	0.0060
	18	18	18	18	17	18	17	18
lhfreq	0.73709	0.89154	0.78894	1.00000	0.33337	0.57026	0.13466	0.52248
	0.0005	<.0001	<.0001		0.1910	0.0135	0.6064	0.0261
	18	18	18	18	17	18	17	18
poatot	0.07923	0.35082	0.30058	0.33337	1.00000	0.14123	0.11514	0.08121
	0.7625	0.1674	0.2411	0.1910		0.5887	0.6599	0.7567
	17	17	17	17	17	17	17	17
sixarctot	0.47141	0.63218	0.52706	0.57026	0.14123	1.00000	-0.15400	0.61380
	0.0483	0.0049	0.0246	0.0135	0.5887		0.5551	0.0067
	18	18	18	18	17	18	17	18
poaden	0.09810	0.10008	0.15677	0.13466	0.11514	-0.15400	1.00000	-0.06460
	0.7080	0.7023	0.5479	0.6064	0.6599	0.5551		0.8054
	17	17	17	17	17	17	17	17
arcden	0.51483	0.56995	0.62101	0.52248	0.08121	0.61380	-0.06460	1.00000
	0.0288	0.0135	0.0060	0.0261	0.7567	0.0067	0.8054	
	18	18	18	18	17	18	17	18

Table C-11. Regression analysis for LH pulse frequency versus BW, POA/ARC cell counts, and POA/ARC cell density in Experiment 1.

The REG Procedure
Model: MODEL1
Dependent Variable: lhfreq

Number of Observations Read	18
Number of Observations Used	17
Number of Observations with Missing Values	1

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	285.50350	57.10070	4.88	0.0135
Error	11	128.73179	11.70289		
Corrected Total	16	414.23529			

Root MSE	3.42095	R-Square	0.6892
Dependent Mean	5.52941	Adj R-Sq	0.5480
Coeff Var	61.86822		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	-16.75009	6.59220	-2.54	0.0274
bw	1	0.28382	0.10547	2.69	0.0210
poatot	1	0.02160	0.01576	1.37	0.1979
sixarctot	1	0.01139	0.00946	1.20	0.2539
poaden	1	0.00045902	0.00081030	0.57	0.5824
arcden	1	0.00031082	0.00109	0.28	0.7812

Table C-11 (cont.).

Model: MODEL1
Dependent Variable: lhfreq

Output Statistics

Obs	Dependent Variable	Predicted Value	Std Error Mean Predict	95% CL Mean		95% CL Predict		Residual
1	6.0000	.	.	.	.	.	.	.
2	2.0000	4.1269	1.2824	1.3043	6.9495	-3.9142	12.1680	-2.1269
3	10.0000	7.7534	1.8499	3.6817	11.8251	-0.8064	16.3133	2.2466
4	9.0000	12.8949	2.2062	8.0391	17.7508	3.9355	21.8544	-3.8949
5	0	1.3224	1.4322	-1.8299	4.4748	-6.8403	9.4852	-1.3224
6	9.0000	7.6207	2.1135	2.9689	12.2724	-1.2298	16.4711	1.3793
7	0	-0.4562	1.9176	-4.6768	3.7645	-9.0879	8.1756	0.4562
8	0	4.4956	1.6530	0.8575	8.1338	-3.8667	12.8580	-4.4956
9	14.0000	9.5824	1.8138	5.5903	13.5745	1.0601	18.1047	4.4176
10	9.0000	5.2836	2.1921	0.4587	10.1084	-3.6591	14.2262	3.7164
11	5.0000	3.7002	2.2930	-1.3468	8.7471	-5.3643	12.7646	1.2998
12	11.0000	10.7861	1.9782	6.4320	15.1402	2.0884	19.4838	0.2139
13	14.0000	11.3703	1.9437	7.0923	15.6484	2.7104	20.0302	2.6297
14	3.0000	3.0867	2.7226	-2.9058	9.0791	-6.5364	12.7097	-0.0867
15	0	1.1714	2.0506	-3.3419	5.6847	-7.6071	9.9499	-1.1714
16	2.0000	7.7305	1.2129	5.0609	10.4001	-0.2582	15.7192	-5.7305
17	0	-1.8158	2.5001	-7.3186	3.6870	-11.1417	7.5102	1.8158
18	6.0000	5.3468	2.6403	-0.4645	11.1582	-4.1644	14.8581	0.6532

Output Statistics

Obs	Std Error Residual	Student Residual	-2-1 0 1 2	Cook's D
1	.	.		.
2	3.171	-0.671	| *| |	0.012
3	2.878	0.781	| |* |	0.042
4	2.614	-1.490	| **| |	0.263
5	3.107	-0.426	| | |	0.006
6	2.690	0.513	| |* |	0.027
7	2.833	0.161	| | |	0.002
8	2.995	-1.501	| ***| |	0.114
9	2.901	1.523	| |*** |	0.151
10	2.626	1.415	| |** |	0.233
11	2.539	0.512	| |* |	0.036
12	2.791	0.0766	| | |	0.000
13	2.815	0.934	| |* |	0.069
14	2.071	-0.0418	| | |	0.001
15	2.738	-0.428	| | |	0.017
16	3.199	-1.792	| ***| |	0.077
17	2.335	0.778	| |* |	0.116
18	2.175	0.300	| | |	0.022

Sum of Residuals	0
Sum of Squared Residuals	128.73179
Predicted Residual SS (PRESS)	265.49774

Table C-12. Regression analysis for LH pulse frequency versus ARC cell counts and ARC cell density in Experiment 1.

The REG Procedure
Model: MODEL1
Dependent Variable: lhfreq

Number of Observations Read	18
Number of Observations Used	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	252.61724	84.20575	7.28	0.0035
Error	14	161.82721	11.55909		
Corrected Total	17	414.44444			

Root MSE	3.39987	R-Square	0.6095
Dependent Mean	5.55556	Adj R-Sq	0.5259
Coeff Var	61.19758		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	-11.65105	5.30889	-2.19	0.0456
bw	1	0.29948	0.10282	2.91	0.0114
sixarctot	1	0.01040	0.00886	1.17	0.2602
arcden	1	0.00031850	0.00108	0.29	0.7729

Table C-12 (cont.).

Model: MODEL1
Dependent Variable: lhfreq

Output Statistics

Obs	Dependent Variable	Predicted Value	Std Error Mean Predict	95% CL Mean		95% CL Predict		Residual
1	6.0000	7.9338	1.1960	5.3687	10.4989	0.2038	15.6638	-1.9338
2	2.0000	3.4006	1.1924	0.8431	5.9580	-4.3269	11.1280	-1.4006
3	10.0000	8.1817	1.6331	4.6790	11.6844	0.0921	16.2713	1.8183
4	9.0000	12.4508	2.0627	8.0268	16.8747	3.9217	20.9798	-3.4508
5	0	1.6609	1.2568	-1.0348	4.3566	-6.1134	9.4352	-1.6609
6	9.0000	8.7585	1.8411	4.8098	12.7071	0.4660	17.0509	0.2415
7	0	0.0190	1.7126	-3.6542	3.6921	-8.1459	8.1838	-0.0190
8	0	4.4466	1.1190	2.0467	6.8466	-3.2301	12.1234	-4.4466
9	14.0000	10.3156	1.6733	6.7269	13.9044	2.1884	18.4429	3.6844
10	9.0000	2.4347	1.2971	-0.3473	5.2168	-5.3699	10.2394	6.5653
11	5.0000	5.4358	1.5601	2.0897	8.7820	-2.5873	13.4589	-0.4358
12	11.0000	9.3903	1.7195	5.7024	13.0781	1.2188	17.5618	1.6097
13	14.0000	9.4479	1.5114	6.2063	12.6895	1.4679	17.4279	4.5521
14	3.0000	5.1107	1.7242	1.4127	8.8088	-3.0654	13.2868	-2.1107
15	0	0.4693	1.4259	-2.5889	3.5275	-7.4380	8.3766	-0.4693
16	2.0000	7.2837	1.1750	4.7636	9.8039	-0.4314	14.9989	-5.2837
17	0	-0.4225	1.7271	-4.1267	3.2817	-8.6013	7.7564	0.4225
18	6.0000	3.6826	2.4045	-1.4746	8.8398	-5.2488	12.6140	2.3174

Output Statistics

Obs	Std Error Residual	Student Residual	-2-1 0 1 2	Cook's D
1	3.183	-0.608	| *| |	0.013
2	3.184	-0.440	| | |	0.007
3	2.982	0.610	| |* |	0.028
4	2.703	-1.277	| **| |	0.237
5	3.159	-0.526	| *| |	0.011
6	2.858	0.0845	| | |	0.001
7	2.937	-0.0065	| | |	0.000
8	3.210	-1.385	| **| |	0.058
9	2.960	1.245	| |** |	0.124
10	3.143	2.089	| |**** |	0.186
11	3.021	-0.144	| | |	0.001
12	2.933	0.549	| |* |	0.026
13	3.045	1.495	| |** |	0.138
14	2.930	-0.720	| *| |	0.045
15	3.086	-0.152	| | |	0.001
16	3.190	-1.656	| ***| |	0.093
17	2.929	0.144	| | |	0.002
18	2.404	0.964	| |* |	0.233

Sum of Residuals	0
Sum of Squared Residuals	161.82721
Predicted Residual SS (PRESS)	257.58686

Table C-13. Regression analysis for LH pulse frequency versus POA/ARC cell counts, and POA/ARC cell densities in Experiment 1.

The REG Procedure
Model: MODEL1
Dependent Variable: lhfreq

Number of Observations Read	18
Number of Observations Used	17
Number of Observations with Missing Values	1

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	200.75580	50.18895	2.82	0.0732
Error	12	213.47949	17.78996		
Corrected Total	16	414.23529			

Root MSE	4.21781	R-Square	0.4846
Dependent Mean	5.52941	Adj R-Sq	0.3129
Coeff Var	76.27962		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	-11.69600	7.79093	-1.50	0.1591
poatot	1	0.02121	0.01943	1.09	0.2965
sixarctot	1	0.01787	0.01128	1.58	0.1390
poaden	1	0.00087844	0.00098039	0.90	0.3879
arcden	1	0.00125	0.00128	0.98	0.3454

Output Statistics

Obs	Dependent Variable	Predicted Value	Std Error Mean Predict	95% CL Mean		95% CL Predict		Residual
1	6.0000	.	.	.	.	.	.	.
2	2.0000	5.3503	1.4785	2.1290	8.5716	-4.3878	15.0883	-3.3503
3	10.0000	9.0979	2.1961	4.3131	13.8828	-1.2629	19.4588	0.9021
4	9.0000	12.1473	2.6985	6.2678	18.0268	1.2376	23.0570	-3.1473
5	0	3.9339	1.2987	1.1042	6.7636	-5.6817	13.5495	-3.9339
6	9.0000	4.1564	2.0666	-0.3464	8.6591	-6.0773	14.3900	4.8436
7	0	0.9285	2.2776	-4.0340	5.8910	-9.5156	11.3726	-0.9285
8	0	3.0080	1.9206	-1.1767	7.1927	-7.0898	13.1058	-3.0080
9	14.0000	5.4231	1.1702	2.8735	7.9726	-4.1139	14.9600	8.5769
10	9.0000	8.4108	2.2917	3.4175	13.4040	-2.0480	18.8695	0.5892
11	5.0000	4.6577	2.7929	-1.4276	10.7430	-6.3642	15.6797	0.3423
12	11.0000	12.1603	2.3564	7.0262	17.2944	1.6336	22.6870	-1.1603
13	14.0000	9.1932	2.1790	4.4456	13.9409	-1.1505	19.5370	4.8068
14	3.0000	2.1022	3.3264	-5.1454	9.3498	-9.6017	13.8061	0.8978
15	0	3.3469	2.3235	-1.7155	8.4093	-7.1451	13.8388	-3.3469
16	2.0000	6.4149	1.3686	3.4330	9.3968	-3.2466	16.0764	-4.4149
17	0	1.0695	2.7847	-4.9977	7.1368	-9.9425	12.0816	-1.0695
18	6.0000	2.5990	3.0021	-3.9419	9.1400	-8.6809	13.8790	3.4010

Table C-13 (cont.).

Output Statistics

Obs	Std Error Residual	Student Residual	-2-1 0 1 2	Cook's D
1	.	.		.
2	3.950	-0.848	*|	0.020
3	3.601	0.251	|	0.005
4	3.242	-0.971	*|	0.131
5	4.013	-0.980	*|	0.020
6	3.677	1.317	|**	0.110
7	3.550	-0.262	|	0.006
8	3.755	-0.801	*|	0.034
9	4.052	2.117	|****	0.075
10	3.541	0.166	|	0.002
11	3.161	0.108	|	0.002
12	3.498	-0.332	|	0.010
13	3.611	1.331	|**	0.129
14	2.593	0.346	|	0.039
15	3.520	-0.951	*|	0.079
16	3.990	-1.107	**|	0.029
17	3.168	-0.338	|	0.018
18	2.963	1.148	|**	0.271

Sum of Residuals	0
Sum of Squared Residuals	213.47949
Predicted Residual SS (PRESS)	357.57761

Table C-14. The Means procedure for variables sorted by high and low LH pulse frequency in Experiment 1.

The MEANS Procedure

freq12	N Obs	Variable	Mean	Std Dev	Std Error
1	9	bw	40.2222222	7.8066923	2.6022308
		lh	1.1333333	0.3840573	0.1280191
		lhfreq	1.3333333	1.8027756	0.6009252
		lhamp	0.6666667	0.9420722	0.3140241
		poatot	142.2222222	50.7956144	16.9318715
		sixarctot	105.0000000	43.6548966	14.5516322
		poaden	4666.78	1311.49	437.1645945
		arcden	4891.44	853.4720428	284.4906809
2	9	bw	51.4444444	8.0329184	2.6776395
		lh	3.4000000	1.2874393	0.4291464
		lhfreq	9.7777778	2.9059326	0.9686442
		lhamp	2.1888889	0.9386752	0.3128917
		poatot	198.7500000	46.0860995	16.2938968
		sixarctot	243.1111111	136.0729992	45.3576664
		poaden	4736.63	893.3098866	315.8327393
		arcden	5594.44	1097.15	365.7165763

Table C-15. Statistical analysis of body weight sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: bw

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	566.722222	566.722222	9.03	0.0084
Error	16	1003.777778	62.736111		
Corrected Total	17	1570.500000			

R-Square	Coeff Var	Root MSE	bw Mean
0.360855	17.28134	7.920613	45.83333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	566.7222222	566.7222222	9.03	0.0084

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	566.7222222	566.7222222	9.03	0.0084

Least Squares Means

freq12	bw LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	40.2222222	2.6402044	<.0001	0.0084
2	51.4444444	2.6402044	<.0001	

Table C-16. Statistical analysis of mean circulating concentrations of LH sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: lh

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	23.12000000	23.12000000	25.62	0.0001
Error	16	14.44000000	0.90250000		
Corrected Total	17	37.56000000			

R-Square	Coeff Var	Root MSE	lh Mean
0.615548	41.91176	0.950000	2.266667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	23.12000000	23.12000000	25.62	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	23.12000000	23.12000000	25.62	0.0001

t Tests (LSD) for lh

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.9025
Critical Value of t	2.11991
Least Significant Difference	0.9494

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	3.4000	9	2
B	1.1333	9	1

Least Squares Means

freq12	lh LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	1.13333333	0.31666667	0.0025	0.0001
2	3.40000000	0.31666667	<.0001	

Table C-17. Statistical analysis of LH pulse amplitude sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: lhamp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	10.42722222	10.42722222	11.79	0.0034
Error	16	14.14888889	0.88430556		
Corrected Total	17	24.57611111			

R-Square	Coeff Var	Root MSE	lhamp Mean
0.424283	65.86286	0.940375	1.427778

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	10.42722222	10.42722222	11.79	0.0034

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	10.42722222	10.42722222	11.79	0.0034

t Tests (LSD) for lhamp

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.884306
Critical Value of t	2.11991
Least Significant Difference	0.9397

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	2.1889	9	2
B	0.6667	9	1

Least Squares Means

freq12	lhamp LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1= LSMean2 Pr > |t|
1	0.66666667	0.31345841	0.0493	0.0034
2	2.18888889	0.31345841	<.0001	

Table C-18. Statistical analysis of POA cell counts sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: poatot

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	13533.41503	13533.41503	5.72	0.0304
Error	15	35509.05556	2367.27037		
Corrected Total	16	49042.47059			

R-Square	Coeff Var	Root MSE	poatot Mean
0.275953	28.81980	48.65460	168.8235

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	13533.41503	13533.41503	5.72	0.0304

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	13533.41503	13533.41503	5.72	0.0304

t Tests (LSD) for poatot

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	2367.27
Critical Value of t	2.13145
Least Significant Difference	50.391
Harmonic Mean of Cell Sizes	8.470588

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	198.75	8	2
B	142.22	9	1

Table C-18 (cont.).

Least Squares Means

freq12	poatot LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1= LSMean2 Pr > |t|
1	142.222222	16.218201	<.0001	0.0304
2	198.750000	17.202000	<.0001	

Table C-19. Statistical analysis of POA cell density sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: poaden

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	20662.45	20662.45	0.02	0.9010
Error	15	19346145.43	1289743.03		
Corrected Total	16	19366807.88			

R-Square	Coeff Var	Root MSE	poaden Mean
0.001067	24.16498	1135.669	4699.647

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	20662.45180	20662.45180	0.02	0.9010

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	20662.45180	20662.45180	0.02	0.9010

t Tests (LSD) for poaden

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1289743
Critical Value of t	2.13145
Least Significant Difference	1176.2
Harmonic Mean of Cell Sizes	8.470588

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	4736.6	8	2
A			
A	4666.8	9	1

Table C-19 (cont.).

Least Squares Means

freq12	poaden LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	4666.77778	378.55618	<.0001	0.9010
2	4736.62500	401.51946	<.0001	

Table C-20. Statistical analysis of ARC cell counts sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: sixarctot

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	85836.0556	85836.0556	8.41	0.0105
Error	16	163372.8889	10210.8056		
Corrected Total	17	249208.9444			

R-Square	Coeff Var	Root MSE	sixarctot Mean
0.344434	58.05533	101.0485	174.0556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	85836.05556	85836.05556	8.41	0.0105

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	85836.05556	85836.05556	8.41	0.0105

t Tests (LSD) for sixarctot

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	10210.81
Critical Value of t	2.11991
Least Significant Difference	100.98

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	243.11	9	2
B	105.00	9	1

Table C-20 (cont.).

Least Squares Means

freq12	sixarctot LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	105.000000	33.682844	0.0066	0.0105
2	243.111111	33.682844	<.0001	

Table C-21. Statistical analysis of ARC cell counts sorted by high and low LH pulse frequency in Experiment 1.

The GLM Procedure

Dependent Variable: arcden

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2223940.50	2223940.50	2.30	0.1487
Error	16	15457216.44	966076.03		
Corrected Total	17	17681156.94			

R-Square	Coeff Var	Root MSE	arcden Mean
0.125780	18.74694	982.8917	5242.944

Source	DF	Type I SS	Mean Square	F Value	Pr > F
freq12	1	2223940.500	2223940.500	2.30	0.1487

Source	DF	Type III SS	Mean Square	F Value	Pr > F
freq12	1	2223940.500	2223940.500	2.30	0.1487

t Tests (LSD) for arcden

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	966076
Critical Value of t	2.11991
Least Significant Difference	982.24

Means with the same letter are not significantly different.

t Grouping	Mean	N	freq12
A	5594.4	9	2
A			
A	4891.4	9	1

Table C-21 (cont.).

Least Squares Means

freq12	arcden LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1= LSMean2 Pr > |t|
1	4891.44444	327.63056	<.0001	0.1487
2	5594.44444	327.63056	<.0001	

Table C-22. Statistical analysis of BW for Experiment 2.

The MEANS Procedure

Analysis Variable : BW

TRTMT	N Obs	Mean	Std Dev	Std Error
0	375	43.8200000	8.1469013	3.6434050
1	450	41.3666667	2.4977323	1.0196949

The TTEST Procedure

Statistics

Variable	TRTMT	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err
BW	0	5	33.704	43.82	53.936	4.8811	8.1469	23.411	3.6434
BW	1	6	38.745	41.367	43.988	1.5591	2.4977	6.126	1.0197
BW	Diff (1-2)		-5.411	2.4533	10.318	3.9492	5.7415	10.482	3.4766

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
BW	Pooled	Equal	9	0.71	0.4983
BW	Satterthwaite	Unequal	4.63	0.65	0.5475

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
BW	Folded F	4	5	10.64	0.0232

Table C-23. Statistical analysis for baseline concentrations of LH for Experiment 2.

The MEANS Procedure

Analysis Variable : LHBASE

TRTMT	N Obs	Mean	Std Dev	Std Error
0	375	0.9468000	0.2707780	0.1210956
1	450	1.0106667	0.5287611	0.2158658

The TTEST Procedure

Statistics

Variable	TRTMT	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err
LHBASE	0	5	0.6106	0.9468	1.283	0.1622	0.2708	0.7781	0.1211
LHBASE	1	6	0.4558	1.0107	1.5656	0.3301	0.5288	1.2968	0.2159
LHBASE	Diff (1-2)		-0.658	-0.064	0.5299	0.2982	0.4335	0.7914	0.2625

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
LHBASE	Pooled	Equal	9	-0.24	0.8132
LHBASE	Satterthwaite	Unequal	7.69	-0.26	0.8032

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
LHBASE	Folded F	5	4	3.81	0.2190

Table C-24. Statistical analysis for LH pulse frequency by period for Experiment 2.

The MEANS Procedure

Analysis Variable : FREQ

TRTMT	PERIOD	N Obs	Mean	Std Dev	Std Error
0	0	15	.	.	.
	1	120	3.0000000	1.5811388	0.7071068
	2	60	.	.	.
	3	120	2.8000000	0.8366600	0.3741657
	4	60	.	.	.
1	0	18	.	.	.
	1	144	6.0000000	0	0
	2	72	.	.	.
	3	144	2.8333333	3.1251667	1.2758439
	4	72	.	.	.

---------- PERIOD=1 ----------

The TTEST Procedure

Statistics

Variable	TRTMT	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err
FREQ	0	5	1.0368	3	4.9632	0.9473	1.5811	4.5435	0.7071
FREQ	1	6	6	6	6	.	0	.	0
FREQ	Diff (1-2)		-4.444	-3	-1.556	0.725	1.0541	1.9244	0.6383

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
FREQ	Pooled	Equal	9	-4.70	0.0011
FREQ	Satterthwaite	Unequal	4	-4.24	0.0132

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
FREQ	Folded F	4	5	Infty	<.0001

Table C-24 (cont.).

(For Period 3, Deleted 8140, 820, 8116, 8124. Only 2 treated ewes, 5 controls)

PERIOD=3

The TTEST Procedure

Statistics

Variable	TRTMT	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err
FREQ	0	5	1.7611	2.8	3.8389	0.5013	0.8367	2.4042	0.3742
FREQ	1	2	6	6	6	.	0	.	0
FREQ	Diff (1-2)		-4.809	-3.2	-1.591	0.4671	0.7483	1.8354	0.6261

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
FREQ	Pooled	Equal	5	-5.11	0.0037
FREQ	Satterthwaite	Unequal	4	-8.55	0.0010

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
FREQ	Folded F	4	1	Infty	<.0001

Table C-25. Statistical analysis for circulating concentrations of LH for Experiment 2.

The MEANS Procedure

Analysis Variable : LH

TRTMT	PERIOD	N Obs	Mean	Std Dev	Std Error
0	0	15	2.4200000	1.7005041	0.4390683
	1	120	2.2491667	1.4367327	0.1311552
	2	60	2.4883333	1.5200459	0.1962371
	3	120	2.3091667	1.2302214	0.1123033
	4	60	2.6966667	1.7489916	0.2257938
1	0	18	1.5055556	1.0389317	0.2448786
	1	144	4.5298611	2.5459986	0.2121666
	2	72	2.1111111	1.3786660	0.1624773
	3	144	5.5166667	6.6841112	0.5570093
	4	72	2.6750000	3.8248170	0.4507590

Table C-25 (cont.).

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Compound Symmetry
Subject Effect	EWEID(TRTMT)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1
PERIOD	5	0 1 2 3 4

Dimensions

Covariance Parameters	2
Columns in X	18
Columns in Z	0
Subjects	11
Max Obs Per Subject	75

Number of Observations

Number of Observations Read	825
Number of Observations Used	825
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	4338.10239198	
1	1	4183.11949786	0.00000000

Convergence criteria met.

Table C-25 (cont.).

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	EWEID(TRTMT)	2.7754
Residual		9.1011

Fit Statistics

-2 Res Log Likelihood	4183.1
AIC (smaller is better)	4187.1
AICC (smaller is better)	4187.1
BIC (smaller is better)	4187.9

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	154.98	<.0001

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	9	0.64	0.4456
PERIOD	4	36	9.15	<.0001
TRTMT*PERIOD	4	36	12.76	<.0001

Least Squares Means

Effect	TRTMT	PERIOD	Estimate	Standard Error	DF	t Value	Pr > |t|
TRTMT*PERIOD	0	0	2.4200	1.0779	36	2.25	0.0310
TRTMT*PERIOD	0	1	2.2492	0.7943	36	2.83	0.0075
TRTMT*PERIOD	0	2	2.4883	0.8407	36	2.96	0.0054
TRTMT*PERIOD	0	3	2.3092	0.7943	36	2.91	0.0062
TRTMT*PERIOD	0	4	2.6967	0.8407	36	3.21	0.0028
TRTMT*PERIOD	1	0	1.5056	0.9840	36	1.53	0.1347
TRTMT*PERIOD	1	1	4.5299	0.7251	36	6.25	<.0001
TRTMT*PERIOD	1	2	2.1111	0.7674	36	2.75	0.0092
TRTMT*PERIOD	1	3	5.5167	0.7251	36	7.61	<.0001
TRTMT*PERIOD	1	4	2.6750	0.7674	36	3.49	0.0013

Table C-25 (cont.).

Differences of Least Squares Means

Effect	TRTMT	PERIOD	_TRTMT	_PERIOD	Estimate	Standard Error	DF	t Value	Pr > |t|
TRTMT*PERIOD	0	0	0	1	0.1708	0.8262	36	0.21	0.8374
TRTMT*PERIOD	0	0	0	2	-0.06833	0.8709	36	-0.08	0.9379
TRTMT*PERIOD	0	0	0	3	0.1108	0.8262	36	0.13	0.8940
TRTMT*PERIOD	0	0	0	4	-0.2767	0.8709	36	-0.32	0.7526
TRTMT*PERIOD	0	0	1	0	0.9144	1.4595	36	0.63	0.5349
TRTMT*PERIOD	0	0	1	1	-2.1099	1.2991	36	-1.62	0.1131
TRTMT*PERIOD	0	0	1	2	0.3089	1.3232	36	0.23	0.8167
TRTMT*PERIOD	0	0	1	3	-3.0967	1.2991	36	-2.38	0.0225
TRTMT*PERIOD	0	0	1	4	-0.2550	1.3232	36	-0.19	0.8483
TRTMT*PERIOD	0	1	0	2	-0.2392	0.4770	36	-0.50	0.6191
TRTMT*PERIOD	0	1	0	3	-0.06000	0.3895	36	-0.15	0.8784
TRTMT*PERIOD	0	1	0	4	-0.4475	0.4770	36	-0.94	0.3544
TRTMT*PERIOD	0	1	1	0	0.7436	1.2646	36	0.59	0.5602
TRTMT*PERIOD	0	1	1	1	-2.2807	1.0755	36	-2.12	0.0409
TRTMT*PERIOD	0	1	1	2	0.1381	1.1045	36	0.12	0.9012
TRTMT*PERIOD	0	1	1	3	-3.2675	1.0755	36	-3.04	0.0044
TRTMT*PERIOD	0	1	1	4	-0.4258	1.1045	36	-0.39	0.7021
TRTMT*PERIOD	0	2	0	3	0.1792	0.4770	36	0.38	0.7094
TRTMT*PERIOD	0	2	0	4	-0.2083	0.5508	36	-0.38	0.7075
TRTMT*PERIOD	0	2	1	0	0.9828	1.2942	36	0.76	0.4526
TRTMT*PERIOD	0	2	1	1	-2.0415	1.1102	36	-1.84	0.0742
TRTMT*PERIOD	0	2	1	2	0.3772	1.1383	36	0.33	0.7423
TRTMT*PERIOD	0	2	1	3	-3.0283	1.1102	36	-2.73	0.0098
TRTMT*PERIOD	0	2	1	4	-0.1867	1.1383	36	-0.16	0.8707
TRTMT*PERIOD	0	3	0	4	-0.3875	0.4770	36	-0.81	0.4219
TRTMT*PERIOD	0	3	1	0	0.8036	1.2646	36	0.64	0.5291
TRTMT*PERIOD	0	3	1	1	-2.2207	1.0755	36	-2.06	0.0462
TRTMT*PERIOD	0	3	1	2	0.1981	1.1045	36	0.18	0.8587
TRTMT*PERIOD	0	3	1	3	-3.2075	1.0755	36	-2.98	0.0051
TRTMT*PERIOD	0	3	1	4	-0.3658	1.1045	36	-0.33	0.7424
TRTMT*PERIOD	0	4	1	0	1.1911	1.2942	36	0.92	0.3635
TRTMT*PERIOD	0	4	1	1	-1.8332	1.1102	36	-1.65	0.1074
TRTMT*PERIOD	0	4	1	2	0.5856	1.1383	36	0.51	0.6101
TRTMT*PERIOD	0	4	1	3	-2.8200	1.1102	36	-2.54	0.0155
TRTMT*PERIOD	0	4	1	4	0.02167	1.1383	36	0.02	0.9849
TRTMT*PERIOD	1	0	1	1	-3.0243	0.7542	36	-4.01	0.0003
TRTMT*PERIOD	1	0	1	2	-0.6056	0.7950	36	-0.76	0.4512
TRTMT*PERIOD	1	0	1	3	-4.0111	0.7542	36	-5.32	<.0001
TRTMT*PERIOD	1	0	1	4	-1.1694	0.7950	36	-1.47	0.1500
TRTMT*PERIOD	1	1	1	2	2.4188	0.4354	36	5.55	<.0001
TRTMT*PERIOD	1	1	1	3	-0.9868	0.3555	36	-2.78	0.0087
TRTMT*PERIOD	1	1	1	4	1.8549	0.4354	36	4.26	0.0001
TRTMT*PERIOD	1	2	1	3	-3.4056	0.4354	36	-7.82	<.0001
TRTMT*PERIOD	1	2	1	4	-0.5639	0.5028	36	-1.12	0.2695
TRTMT*PERIOD	1	3	1	4	2.8417	0.4354	36	6.53	<.0001

Table C-26. Statistical analysis of mean circulating concentrations of LH during Period 0 in Experiment 2.

---------- PERIOD=0 ----------

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1

Dimensions

Covariance Parameters	2
Columns in X	3
Columns in Z	11
Subjects	1
Max Obs Per Subject	33

Number of Observations

Number of Observations Read	33
Number of Observations Used	33
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	113.43503244	
1	1	107.25460034	0.00000000

Convergence criteria met.

Table C-26 (cont.).

---------- PERIOD=0 ----------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
EWEID	0.9585
Residual	1.0630

Fit Statistics

-2 Res Log Likelihood	107.3
AIC (smaller is better)	111.3
AICC (smaller is better)	111.7
BIC (smaller is better)	112.1

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	22	1.74	0.2011

Table C-27. Statistical analysis of mean circulating concentrations of LH during Period 1 in Experiment 2.

---------- PERIOD=1 ----------

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1

Dimensions

Covariance Parameters	2
Columns in X	3
Columns in Z	11
Subjects	1
Max Obs Per Subject	264

Number of Observations

Number of Observations Read	264
Number of Observations Used	264
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	1145.91914752	
1	1	1078.13165924	0.00000000

Convergence criteria met.

Table C-27 (cont.).

---------- PERIOD=1 ----------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
EWEID	1.5918
Residual	3.1632

Fit Statistics

-2 Res Log Likelihood	1078.1
AIC (smaller is better)	1082.1
AICC (smaller is better)	1082.2
BIC (smaller is better)	1082.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	253	8.23	0.0045

Table C-28. Statistical analysis of mean circulating concentrations of LH during Period 2 in Experiment 2.

---------- PERIOD=2 ----------

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1

Dimensions

Covariance Parameters	2
Columns in X	3
Columns in Z	11
Subjects	1
Max Obs Per Subject	132

Number of Observations

Number of Observations Read	132
Number of Observations Used	132
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	472.92186434	
1	1	419.24047705	0.00000000

Convergence criteria met.

Table C-28 (cont.).

---------- PERIOD=2 ----------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
EWEID	1.1169
Residual	1.1589

Fit Statistics

-2 Res Log Likelihood	419.2
AIC (smaller is better)	423.2
AICC (smaller is better)	423.3
BIC (smaller is better)	424.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	121	0.32	0.5728

Table C-29. Statistical analysis of mean circulating concentrations of LH during Period 3 in Experiment 2.

---------- PERIOD=3 ----------

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1

Dimensions

Covariance Parameters	2
Columns in X	3
Columns in Z	11
Subjects	1
Max Obs Per Subject	264

Number of Observations

Number of Observations Read	264
Number of Observations Used	264
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	1597.38386369	
1	1	1400.86971322	0.00000000

Convergence criteria met.

Table C-29 (cont.).

---------- PERIOD=3 ----------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
EWEID	17.7775
Residual	10.4161

Fit Statistics

-2 Res Log Likelihood	1400.9
AIC (smaller is better)	1404.9
AICC (smaller is better)	1404.9
BIC (smaller is better)	1405.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	253	1.54	0.2157

Table C-30. Statistical analysis of mean circulating concentrations of LH during Period 4 in Experiment 2.

---------- PERIOD=4 ----------

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJ
Dependent Variable	LH
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
EWEID	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
TRTMT	2	0 1

Dimensions

Covariance Parameters	2
Columns in X	3
Columns in Z	11
Subjects	1
Max Obs Per Subject	132

Number of Observations

Number of Observations Read	132
Number of Observations Used	132
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	668.28420539	
1	1	662.19912782	0.00000000

Convergence criteria met.

Table C-30 (cont.).

---------- PERIOD=4 ----------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
EWEID	1.2617
Residual	8.3299

Fit Statistics

-2 Res Log Likelihood	662.2
AIC (smaller is better)	666.2
AICC (smaller is better)	666.3
BIC (smaller is better)	667.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TRTMT	1	121	0.00	0.9796

Table C-31. Statistical analysis for LH pulse amplitude for Experiment 2.

The MEANS Procedure

Analysis Variable : Amp

Trt	N Obs	Mean	Std Dev	Std Error
0	19	3.0533684	1.8683089	0.4286195
1	36	5.2664444	2.1455901	0.3575984

The Mixed Procedure

Model Information

Data Set	WORK.KISSINJAMP
Dependent Variable	Amp
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
Ewe	11	347 359 368 820 8110 8116 8124 8130 8133 8140 8157
Trt	2	0 1

Dimensions

Covariance Parameters	1
Columns in X	3
Columns in Z	0
Subjects	1
Max Obs Per Subject	55

Number of Observations

Number of Observations Read	55
Number of Observations Used	55
Number of Observations Not Used	0

Table C-31 (cont.).

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	4.2256

Fit Statistics

-2 Res Log Likelihood	233.3
AIC (smaller is better)	235.3
AICC (smaller is better)	235.4
BIC (smaller is better)	237.3

The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Trt	1	53	14.41	0.0004

Table C-32. Statistical analysis of progesterone 5 days post treatment in Experiment 2.

Table of TRT by PostD5

TRT	PostD5		
Frequency, Percent, Row Pct, Col Pct	0	1	Total
0	5 45.45 100.00 71.43	0 0.00 0.00 0.00	5 45.45
1	2 18.18 33.33 28.57	4 36.36 66.67 100.00	6 54.55
Total	7 63.64	4 36.36	11 100.00

Statistics for Table of TRT by PostD5

Statistic	DF	Value	Prob
Chi-Square	1	5.2381	0.0221
Likelihood Ratio Chi-Square	1	6.7824	0.0092
Continuity Adj. Chi-Square	1	2.7533	0.0971
Mantel-Haenszel Chi-Square	1	4.7619	0.0291
Phi Coefficient		0.6901	
Contingency Coefficient		0.5680	
Cramer's V		0.6901	

WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	5
Left-sided Pr <= F	1.0000
Right-sided Pr >= F	0.0455
Table Probability (P)	0.0455
Two-sided Pr <= P	0.0606

Sample Size = 11

Table C-33. Statistical analysis of onset of cyclicity in Experiment 2.

Table of TRT by Chi35

TRT	Chi35		
Frequency, Percent, Row Pct, Col Pct	0	1	Total
0	2 18.18 40.00 50.00	3 27.27 60.00 42.86	5 45.45
1	2 18.18 33.33 50.00	4 36.36 66.67 57.14	6 54.55
Total	4 36.36	7 63.64	11 100.00

Statistics for Table of TRT by Chi35

Statistic	DF	Value	Prob
Chi-Square	1	0.0524	0.8190
Likelihood Ratio Chi-Square	1	0.0523	0.8191
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.0476	0.8273
Phi Coefficient		0.0690	
Contingency Coefficient		0.0688	
Cramer's V		0.0690	

WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	2
Left-sided Pr <= F	0.8030
Right-sided Pr >= F	0.6515
Table Probability (P)	0.4545
Two-sided Pr <= P	1.0000

Sample Size = 11

Table C-33 (cont.).

The TTEST Procedure

Statistics

Variable	TRT	N	Lower CL Mean	Mean	Upper CL Mean	Lower CL Std Dev	Std Dev	Upper CL Std Dev	Std Err
Cycle	0	3	26.539	30.333	34.128	0.7953	1.5275	9.6001	0.8819
Cycle	1	5	28.738	32.8	36.862	1.9598	3.2711	9.3996	1.4629
Cycle	Diff (1-2)		-7.493	-2.467	2.5595	1.8125	2.8127	6.1937	2.0541

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
Cycle	Pooled	Equal	6	-1.20	0.2751
Cycle	Satterthwaite	Unequal	5.88	-1.44	0.1998

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
Cycle	Folded F	4	2	4.59	0.3739

VITA

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